Development of repressible sterility in Medfly for chemical-free pest control

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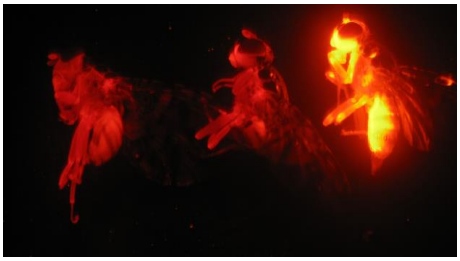

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Summary

Medfly (*Ceratitis capitata*) is an invasive Tephritid fruit fly that severely disrupts global agricultural productivity. Pesticides are the primary control method despite genetic resistance, questionable efficacy, and negative effects upon the environment. The sterile insect technique (SIT) is an ecofriendly alternative, that suppresses the reproduction of wild Medfly by the mass release of sterilised males. Currently, males are sterilised by irradiation, which frequently reduces the ability of males to court females and thereby suppress reproduction.

To address these undesirable effects, we developed a novel sterilisation strategy, based on the tetracycline-repressible expression of a nuclease effector in the male germline. Strains expressing these effectors were 99-100% sterile in the absence of tetracycline, but fertile in the presence of tetracycline. Male mating competitiveness was not detectably reduced in one strain expressing the effector, indicating that these expression systems are suitable for field use. Subsequently, a fluorescent marking system to label sperm was developed, which provided a useful tool to assess the mating competitiveness of sterile males: it was possible to accurately differentiate whether females had mated wild or transgenic males, under field-simulated conditions.

These components may be merged with a tetracycline-repressible genetic switch to remove females from the rearing population, to improve the efficacy of the strategy by releasing sterile males alone. Thereafter, a full assessment of the life history traits of the strain and its mating competitiveness under field-like conditions will be performed, to confirm that the release of these sterile males is capable of suppressing wild populations of Medfly.

Finally, an efficient expression system for CRISPR effectors in the germline was developed. This will facilitate the characterisation of novel transgenic systems for the reproductive control of Medfly, and expedite the development of commercial products, by the targeted integration of transgenic effectors.

Abbreviations

2-pB	Two-ended piggyBac vector
4-pB	Four-ended piggyBac vector
achi	achintya
adh	Alcohol dehydrogenase
Al	*Anastrepha ludens*, Mexican fruit fly
aly	always early
AmCyan	*Anemonia majano* cyan fluorescent protein
APHIS	Animal and Plant Health Inspection Service (USDA)
att	Attachment site
BLAST	Basic local alignment search tool
Bm	*Bombyx mori,* silkworm
bp	Base pairs
BSA	Bovine serum albumin
Bz	*Bactrocera zonata*
CABI	Centre for Agriculture and Bioscience International
can	cannonball
Cas	CRISPR associated
Cas9	CRISPR associated protein 9
Cc	*Ceratitis capitata*, Mediterranean fruit fly
Cc-Rp17S	Medfly ribosomal protein 17S
Cc-RpP0	Medfly ribosomal protein p0
Ccprot	Medfly protamine-like gene
CD	Coding domain
CDC	Center for Disease Control
cDNA	Complementary DNA
CI	Confidence interval
comr	cookie monster
CRISPR	Clustered regularly interspersed palindromic repeats
crRNA	CRISPR RNA
Cry	*Bacillus* species crystalline toxins
Ct	Threshold cycle
CySC	Cyst stem cell
DDT	Dichlorodiphenyltrichloroethane
DE	Downstream enhancer
Dm	*Drosophila melanogaster*
Dmprot	*Drosophila melanogaster* protamine-like
DNA	Deoxyribonucleic acid
DsRed	*Discosoma* species red fluorescent protein
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
FAO	Food and Agriculture Organisation
FLP	Flippase
FokI	*Flavobacterium okeanokoites* restriction enzyme I
FRT	Flippase recombination target
FRT	FLP recombination target
gDNA	Genomic DNA
GFP	Green fluorescent protein

GM	Genetic modification
GMO	Genetically modified organism
GSC	Germline stem cell
GSS	Genetic sexing strain
HCl	Hydrochloric acid
Het	Heterozygous
HMG	High mobility group
Hom	Homozygous
HR	Homologous recombination
hsp	Heat shock protein
IAEA	International Atomic Energy Agency
ITR	Inverted terminal repeat
LB	Luria-Bertrani
lox	Locus of crossing over
MAct	Muscle actin
mCherry	Monomeric *Discosoma* species red fluorescent protein
mia	Meiosis I arrest
min	Minute
minipromoter	Minimal promoter
mip	Myb-interacting protein
Mls	Membrane localisation sequence
mRNA	Messenger RNA
MSA	Multiple sequence alignment
MST	Male specific transcript
n	Sample size
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
NHEJ	Non-homologous end joining
nht	no-hitter
nls	Nuclear localisation sequence
No RT	No reverse transcriptase
NT	Non-tetracycline
nt	Nucleotides
OPIE2	Orgyia pseudotsugata multicapsid nucleopolyhedrosis virus immediate-early 2
ORF	Open reading frame
OX	Oxitec
p	Probability
PAM	Proto-adjacent spacer motif
pB	*piggyBac*
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PhiYellow	*Phialidium* species yellow fluorescent protein
Prot	Protamine
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
R	Replicate

RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNA-seq	High throughput transcript sequencing
RP	Ribosomal protein
rpm	Revolutions per minute
RSI	Relative sterility index
RT-PCR	Reverse transcriptase polymerase chain reaction
RVD	Repeat variable dinucleotide
rye	ryan express
sa	spermatocyte arrest
SDS	Sodium dodecyl sulphate
SG4	Serine-glycine(4) linker
sgRNA	Single guide RNA
SIT	Sterile insect technique
Sp	*Streptococcus pyogenes*
SV40	Simian virus 40
T3	T3 bacteriophage
T7	T7 bacteriophage
TAF	TATA box binding protein associated factor
TALEN	Transcription activator-like effector nuclease
TCE	Translational control element
tet	Tetracycline
tetO	Tetracycline-responsive operator
TG	Transgenic
tMAC	Testis meiotic arrest complex
tomb	tombola
topi	matotopetli
tra	Transformer
tracrRNA	trans-activating CRISPR RNA
Tris	Tris(hydroxymethyl)aminomethane
tsl	Temperature sensitive lethal
TSS	Transcriptional start site
tTAF	Testis TATA box binding protein associated factor
tTAV	Tetracycline-controlled transcriptional activator
UE	Upstream enhancer
UTR	Untranslated region
vis	vismay
WT	wildtype
wuc	wake-up-call
ZFN	Zinc-finger nuclease
zsGreen	*Zoanthus* green fluorescent protein
β2T	β2-tubulin

Table of Contents

Chapter 1 – Introduction

1.1 Pest insects can damage crops and spread disease

The insect class is diverse, comprising over half of all described animal species (Mayhew, 2007). The majority of species (> 99%) do not negatively impact humanity, and perform important roles such as nutrient cycling, pollination, and the production of useful compounds including silk (Sallam, 2013). However, pest species are associated with crop destruction (Bardner and Fletcher, 1974), disease transmission (Lounibos, 2002), and architectural damage (Su, 2002). It is estimated that 14% of food production is lost to insect pests (Strickland, 1969), (Pimentel, 2007). Furthermore, seven neglected tropical diseases transmitted by insects (malaria, trypanosomiasis, lymphatic filiarisis, dengue fever, leishmaniasis, Chagas disease and onchocerciasis) were responsible for 1.23 million deaths, in 2001 alone (World Health Organisation, 2002). Consequently, an effective strategy to selectively limit the populations of pest insect species would improve quality of life, by reducing the incidence of illness and improving the efficiency of food production. This study concerns the development of a novel control strategy for Medfly, based upon the mass-release of males sterilised by the targeted localisation of protamine-FokI nuclease in the male germline. However, several aspects of the methodology and genetic engineering practices could be readily adapted to other pest species, such as disease vectors (Alphey et al., 2010).

Most insect pests are Dipterans (true flies), a diverse order of insects with more than 124000 species described (Merritt et al., 2009). The order includes a variety of important species such as the model organism *D. melanogaster*, the dengue vector *Aedes aegypti* (yellow fever mosquito) and the agricultural pest *Ceratitis capitata* (Medfly). The primary characteristics of this order are a single pair of wings with balancing organs (halteres), compound eyes, well-developed antennae, and a holometabolic life cycle (complete metamorphosis). Two suborders of Diptera are recognised, Nematocera and Brachycera, which are primarily differentiated by the size of the antennae and the body plan (Yeates and Wiegmann, 1999). Nematoceran insects (such as gnats, mosquitoes and midges) are more delicate, with multisegmented antennae. Brachyceran insects, such as Medfly, are smaller, thicker and possess antennae that are comparatively less segmented (Cranston and Gullan, 2009).

1.2 Medfly is a pest of economically important crops

1.2.1 Taxonomy, economic impact and global distribution

Medfly (*Ceratitis capitata*) is a pest fruit fly of the Tephritid family (true fruit flies). This group includes about 4200 species, of which 1400 species lay eggs in fruit (Gesmallah and Abdellah, 2011). Females puncture the skin of the fruit during egg-laying (oviposition), damaging it and increasing susceptibility to infection (Harris et al., 1980). Thereafter, hatching larvae consume the fruit (Wimmer, 2005). Medfly is a globally established pest with the ability to severely reduce agricultural productivity. The species is strongly invasive due to high fecundity, a short life cycle, and the ability to store sperm (Sakai et al., 2001). Furthermore, it tolerates a variety of climates and can oviposit in more than 250 species of fruit (Centers for Disease Control and Prevention, 1999), (Malacrida et al., 2006). This high degree of adaptability and invasiveness makes it one of the most serious Tephritid pests (De Meyer et al., 2007). Medfly continues to impose severe economic consequences, despite successful control programmes in some regions (La Brecque, 1982), (Ehler et al., 1984), (De Longo et al., 2000). The species originated in sub-Saharan Africa, but as a consequence of trade, has spread to have established populations in a variety of equatorial climates in Central America, South America, Europe, Asia, Africa and Australia (Steck et al., 1996), (Centre for Agriculture and Bioscience International, 2015). A map of its current distribution is provided in **Figure 1.1-D**.

1.2.2 Life history

Medfly is a holometabolous insect, progressing through four life stages: egg, larva, pupa and adult (**Figure 1.1-B**). As with most arthropods, development is temperature sensitive, proceeding more slowly at lower temperatures (Briere et al., 1999), (Duyck and Quilici, 2002). Under optimal conditions (28° C), the entire life cycle is approximately 25 days (APHIS, 2015). However, at the lowest permissive temperature for growth and development (about 15° C), the life cycle may take more than 100 days to complete (Department of Agriculture and Food, Western Australia, 2015). Embryos hatch 3-4 days after oviposition. The highly motile larvae burrow through the fruit, and complete three moults (instars), each lasting 3-4 days. Third instar larvae evacuate the fruit, and pupate on the soil surface or slightly beneath the surface. Approximately ten days later, adults eclose and disperse.

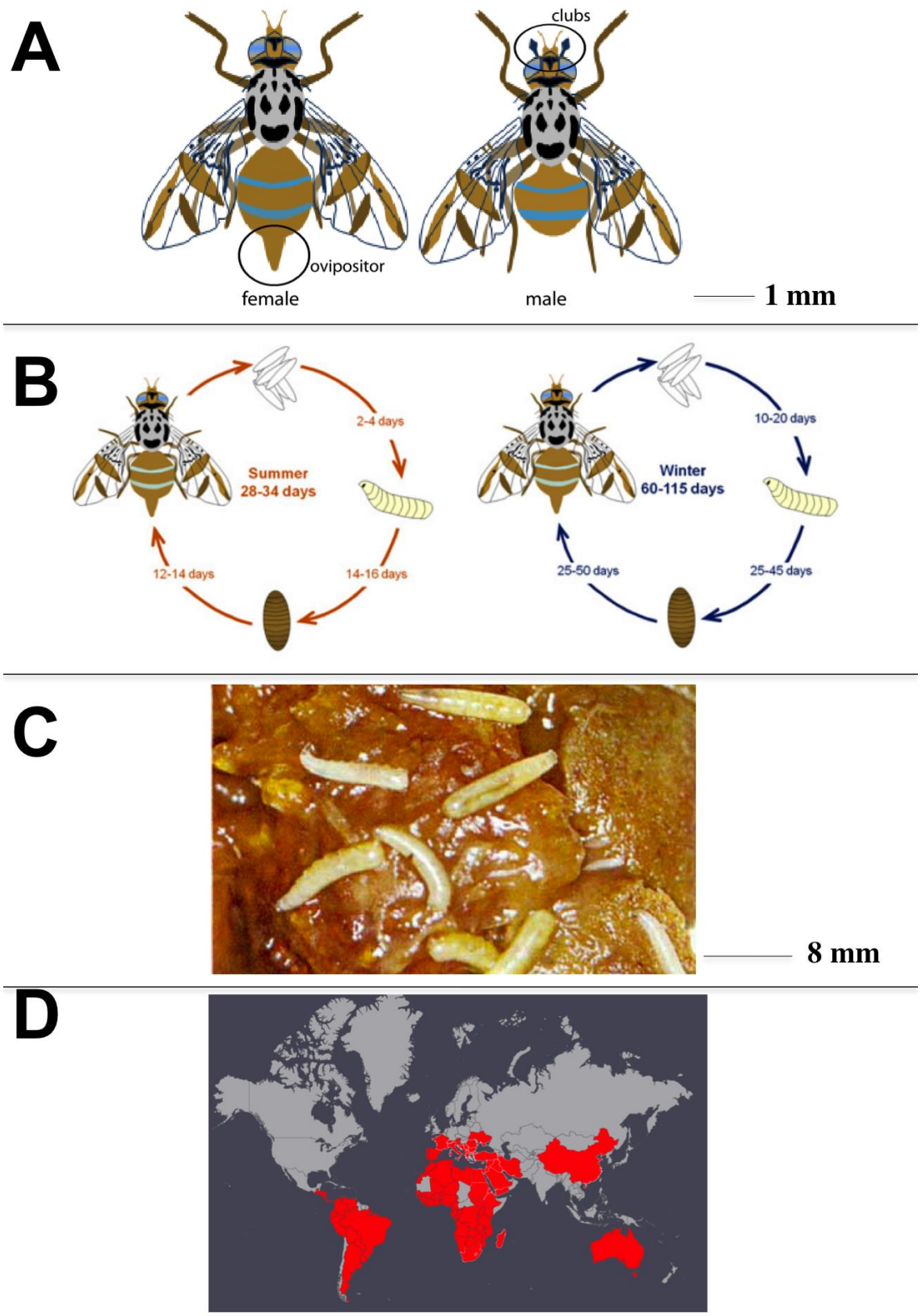


Fig 1.1. The appearance, life cycle and present range of Medfly. (**A**) Sexually dimorphic male and female adult Medfly. Key features for rapid sex identification are highlighted. (**B**) The holometabolous life cycle of Medfly progresses through four life stages: eggs, three larval instars, pupae, and adulthood. Development may take 20-100 days, depending on climate. (**C**) Fruit infested by Medfly larvae. (**D**) Countries reporting Medfly. Note that infestations may be low-level; not all regions of a given country (eg. China) are infested; and that data is missing from some countries. Data from CABI, 2015: http://www.cabi.org/isc/datasheet/12367. Images from Department of Agriculture and Food, Western Australia (A-B) and FDACS-DPI [Florida Department of Agriculture and Consumer Services Division of Plant Industry] (C).

1.2.3 Sexual dimorphism

Males and females are highly dimorphic (Saul, 1982b). Males possess an extra pair of antennae; a white frons (grey in females); black setae; lighter femoral bristles; lighter labial palp bristles; and iridescent eyes. Furthermore, females possess a large ovipositor, absent from males. Males and females begin to accept mates after about three days (Papadopoulos et al., 1998). In terms of sexual anatomy (**Figure 1.2**), males possess a large pair of testes, long tubular accessory glands, short accessory glands, and an ejaculatory duct (Marchini et al., 2003). The male medfly has an intromittent organ with three ejaculatory tracts, which transfer sperm into both spermathecae and the fertilisation chamber of females (Marchini et al., 2001). Large ovaries, accessory glands, a fertilisation chamber and spermathecae comprise the female reproductive tract (Marchini et al., 2001). The long-term storage of sperm in the spermathecae allows the utilisation of sperm from multiple partners (Bertin et al., 2010). These reproductive adaptations make it a highly fecund species; under ideal conditions, a single female can produce hundreds of progeny in her lifetime (Novoseltsev et al., 2004).

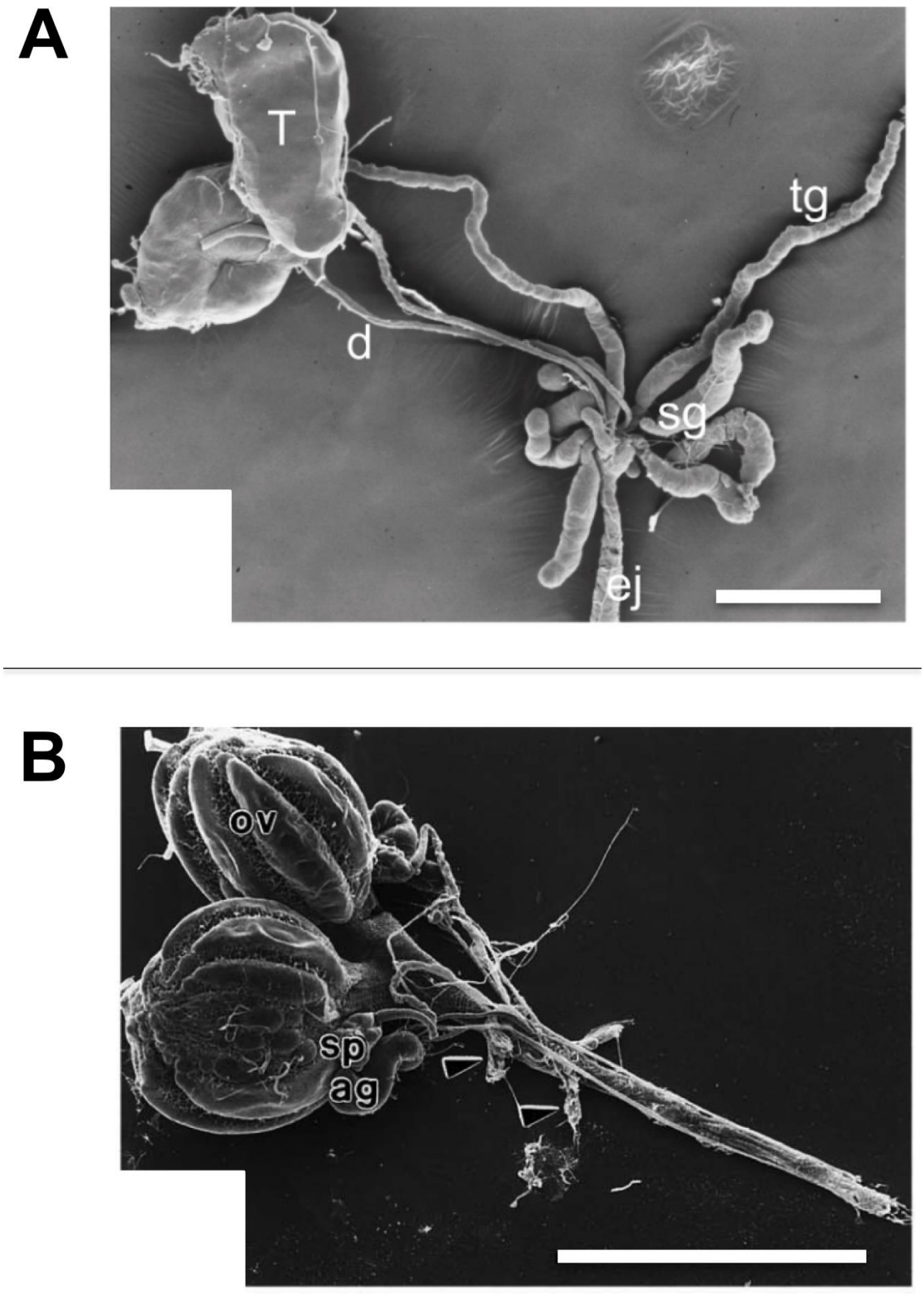


Fig 1.2. Electron micrographs of the male and female reproductive tracts of Medfly. (**A**) Male reproductive tract. Abbreviations: T: testes; d: deferent ducts; tg: long tubular accessory glands (proximal tract); sg: short accessory glands; ej: ejaculatory duct. Scale bars: 250 μm. From Marchini et al (2003). (**B**) Female reproductive tract of Medfly. Abbreviations: ov: ovaries; ag: accessory glands; sp: spermatchae. Scale bars: 250 μm. From Marchini *et al* (2001).

1.2.4 Mating behaviour

The mating behaviour of Medfly is highly complex and involves a strong element of female selection (Kraaijeveld et al., 2005). It is a polygamous species: males and females can mate several times (Miyatake et al., 1999), (Bonizzoni et al., 2002), (Kraaijeveld and Chapman, 2004). Females are more likely to re-mate when transferred insufficient sperm or seminal fluid (Mossinson and Yuval, 2003), (Kraaijeveld and Chapman, 2004). Re-mated females store sperm from both males (Bertin et al., 2010). However, a last-mate precedence is observed for several days after re-mating: a greater proportion of progeny are sired from the most recent mate (Scolari et al., 2014). In *Drosophila,* sperm may incapacitate one another (Price et al., 1999) and females are able to dump sperm (Snook and Hosken, 2004). However, these mechanisms do not seem to be present in Medfly (Bertin et al., 2010).

Females respond to cues of male fitness at several stages. Prior to mating, females respond positively to large symmetrical males that are well-fed (Orozco and Lopez, 1993), (Taylor and Yuval, 1999), (Hunt et al., 2004). Furthermore, males engage in the courtship behavior of lekking. This is a competitive display that establishes dominance within a group, and involves physical, pheromonal and acoustical presentation to females (Field et al., 2002). In this process, the male emits pheromone, bends the abdomen and vibrates his wings in repetitive bursts. Subsequently, the pheromone pouch is closed and a pattern of wing movement initiated. The head is rapidly revolved in patterned movements, prior to attempted copulation. Finally, the male leaps atop the female, repeatedly rubbing with the hind legs and nipping at the ovipositor (Arita and Kaneshiro, 1985). Ultimately, the female commands reproduction, and frequently dislodges males attempting copulation (Briceño and Eberhard, 1998). The process is energetically expensive, and only a minority of lekking Medfly males are able to secure the majority of females for reproduction (Arita and Kaneshiro, 1985). Therefore, it appears that the behaviour is an adaptive mechanism, applying sexual conflict to allow the selection of well-adapted males (Kaspi et al., 2000). After mating, females shift behaviour from mate-seeking to oviposition, in a process thought to be mediated by chemicals in seminal fluid (Jang, 1995), (Gomulski et al., 2012).

Mating behaviour has been observed in the laboratory and in the wild. It is apparent that colonisation and mass-rearing negatively affect the ability of Medfly to engage in wild-type mating behaviour. Several aspects of mating behaviour are reduced

in duration, or removed completely, in colonised Medfly (Liimatainen et al., 1997), (Briceño and Eberhard, 1998). Therefore, it appears that the artificial conditions of rearing limit the highly selective process of mate selection observed in the wild. This is thought to alter the facultative polyandry of wild Medfly (females are highly sexually selective, but occasionally remate) to a condition of scramble polygyny, where males able to quickly secure females are rewarded (Mossinson and Yuval, 2003). As the pest control mechanism described in this thesis applies sterile males to reduce the reproductive capacity of wild populations, the effects of sterilisation and mass-rearing on mating behaviours is of paramount importance.

1.2.5 Spermatogenesis

Spermatogenesis describes the differentiation of primordial germ cells into mature sperm: highly elongated, motile cells capable of fertilisation (Fuller, 1993). To achieve this, tightly regulated and highly specific patterns of gene expression are required in the male germline (Perezgasga et al, 2004). This is relevant to the work performed in this thesis, because we attempted to engineer the expression of effectors for male sterility and fluorescent marking of sperm nuclei, in the male germline. Therefore, the patterns of gene expression in these cells must be considered. The process is well-understood in the model organism *D. melanogaster* (Fuller, 1993), (White-Cooper, Doggett & Ellis, 2008), (White-Cooper, 2009), (Fabian and Brill, 2012). Spermatogenesis in Medfly has been studied, but to a lesser extent (Shahjahan et al., 2006), (Scolari et al., 2008), (Intra et al., 2011), (Papanastasiou et al., 2011), (IAEA/FAO, 2016). To the author's knowledge, there is no single publication that directly compares spermatogenesis in the two organisms. Therefore, the current knowledge of spermatogenesis in *D. melanogaster* and Medfly are compared (**Figure 1.3**).

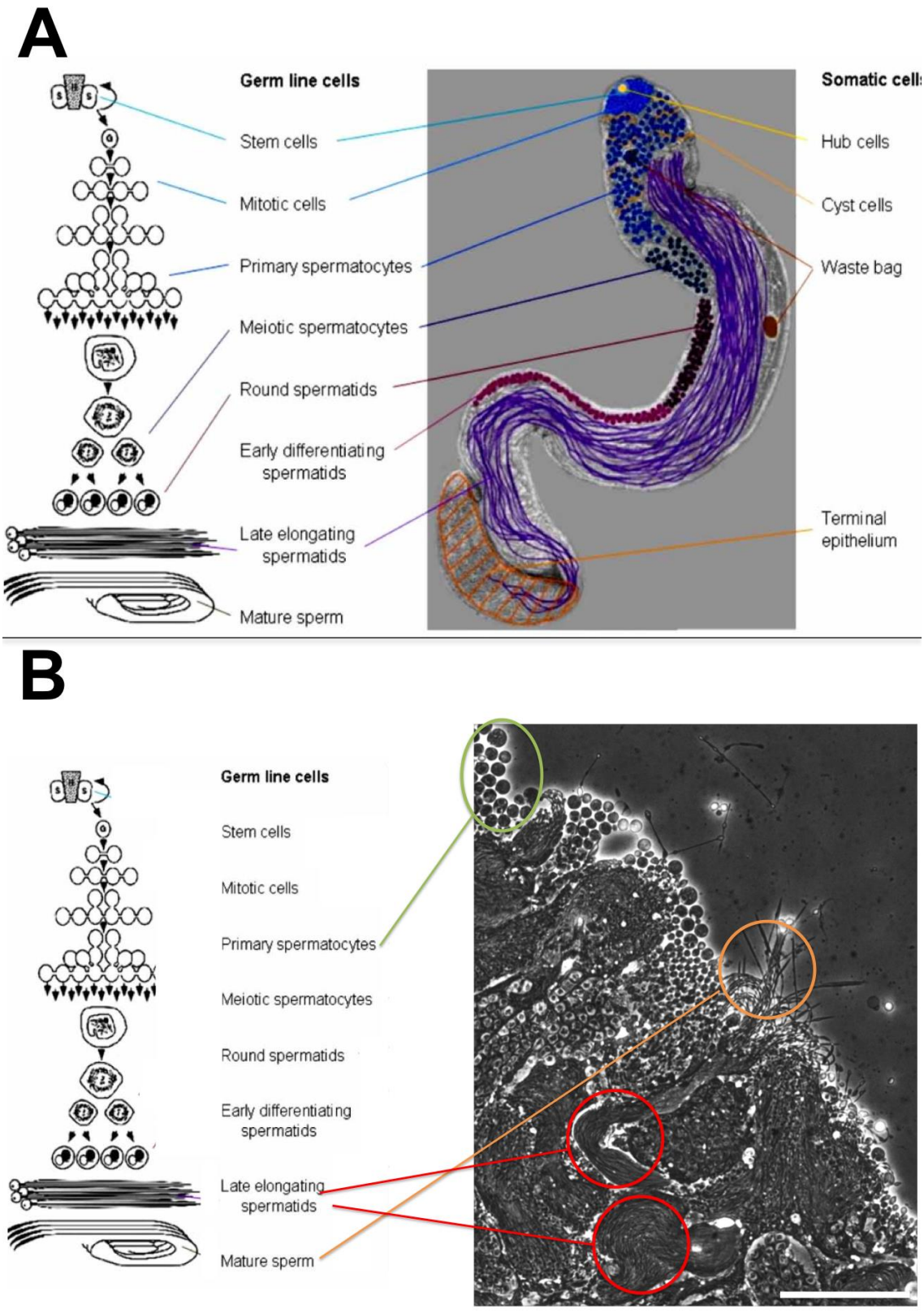


Figure 1.3. Spermatogenesis in *D. melanogaster* and Medfly. (A) Diagram of spermatogenesis in *D. melanogaster*, showing differentiating cells in spermatogenesis. Image from Fuller (1993). (B) Phase contrast microscopy of Medfly testes showing primary spermatocytes, elongating spermatids and mature sperm. Scale bars: 100 μm.

In *D. melanogaster*, a niche of hub cells mediates the cellular signalling required to renew two populations of stem cell essential for spermatogenesis: cyst stem cells (CySC) and germline stem cells (GSCs) (White-Cooper, 2009), (Yamashita, 2010). When CPCs divide, they regenerate and also produce a cyst cell. GSC division regenerates a GSC, but also yields a spermatogonium, which is enveloped by a pair of cyst cells (Leatherman and DiNardo, 2008). The spermatogonium undergoes four mitotic divisions to produce 16 primary spermatocytes (Fuller, 1993). These cells are highly transcriptionally active, in contrast to post-meiotic cells, which demonstrate highly reduced transcription (Chen, 2005), (Vibranovski et al., 2010). The importance of the meiotic transcriptional arrest is discussed in the context of this study (**Section 1.4.2.3**), when the functional components of the male sterility expression system applied in this thesis are considered. Thereafter, two meiotic divisions yield 64 spermatids, which do not immediately separate but remain interconnected via cytoplasmic bridges (Fabian and Brill, 2012). Finally, the spermatid tails elongate, fully differentiated spermatids individualise, and mature sperm migrate to the seminal vesicle (White-Cooper, 2009).

The high degree of differentiation observed in spermatogenesis is reflected by complex patterns of gene expression. At least 1317 genes are uniquely expressed, and at least 2079 are predominantly expressed, in *D. melanogaster* testes (Chintapalli et al., 2007). Transcription of testis-specific genes occurs mostly in primary spermatocytes (Chen, 2005), (Vibranovski et al., 2010). Many transcripts are directly translated in spermatocytes, for example, *β2-tubulin* (Michiels et al., 1989) and *aly* (White-Cooper et al., 2000). Interestingly, the translation of many genes is repressed until the spermatid stage, for example the sperm tail protein Don juan and the protamine-like genes Mst35Ba and Mst35Bb (Santel et al., 1997), (Jayaramaiah Raja and Renkawitz-Pohl, 2005), (White-Cooper, 2009).

Testis-specific transcription in *D. melanogaster* is frequently, but not always, regulated by the meiotic arrest genes. Males with homozygous loss of function mutations for the meiotic arrest genes are not fertile, and accumulate primary spermatocytes, with no subsequent differentiation into spermatids or mature spermatozoa (Lin et al., 1996). The first class of mutants are genes whose protein products are associated with the testis-specific meiotic arrest complex (tMAC). The protein products of *aly, comr, mip40*, *tomb* and *topi* are subunits of tMAC, whereas

those of *achi/vis* and *wuc* associate with tMAC subunits (Perezgasga et al, 2004), (Beall et al., 2007), (Jiang et al., 2007), (Doggett et al., 2011). In the testes of *aly* mutants, 1000 genes are underexpressed by a factor of 16 or more, relative to wild type (Doggett et al., 2011). Therefore, tMAC is a master regulator of transcription in the male germline.

The second class of mutants are genes paralogous to TATA-binding protein associated factors (TAFs): *can, mia*, *nht*, *rye* and *sa*. It is hypothesised that these comprise testis-specific versions of the basal transcription factor TFIID, termed tTAFs (Hiller et al., 2001), (Hiller et al., 2004). Like tMAC mutant testes, tTAF mutants demonstrate a reduction in the expression of testis-specifically transcribed genes, but the effect is less pronounced (White-Cooper, Schafer et al. 1998), (Doggett et al., 2011). From a comparison of the genes underexpressed in tMAC or tTAF mutants, two observations followed. First, tTAF-dependent genes are a subset of tMAC-dependent genes. Secondly, the loss of tMAC subunits or their associated factors almost completely removes transcription of the dependent genes; the loss of tTAFs is less severe, reducing expression of dependent genes to basal levels.

It is not yet understood how tMAC and tTAFs activate the transcription of target genes. DNA-binding domains are present in several tMAC subunits or their associated proteins, including Achi/vis, comr, tomb and topi (Attrill, Falls et al. 2016). Furthermore, tMAC subunits and tTAFs are enriched on chromatin (Chen, 2005), (Jiang et al., 2007), (Metcalf and Wassarman, 2007). A variety of testis-specific promoters are known in *D. melanogaster*, and in several instances, the elements responsible for testis-specific expression have been characterised by reporter study. Motifs that are well-characterised include the 14 bp region upstream of *β2-tubulin* (β2UE1), essential for testis-specific expression and conferring tTAF-sensitivity (Michiels et al., 1989), (Hiller et al., 2001). Two additional elements, one within the promoter (β2UE2) and one within the 5'UTR (β2DE1), enhance expression (Michiels et al., 1993), (Santel, 2000). Homologues of these elements exist in the Medfly *β2-tubulin* homologue (Scolari et al., 2008), though their ability to confer testis-specific transcription has not been confirmed directly. Similarly, a 10 bp translational control sequence (TCE) is present in certain testis-specifically expressed genes, and responds to tTAF (Kempe, Muhs et al. 1993), (Katzenberger et al., 2012). Interestingly, replacement of the β2UE1 sequence with a TCE retains the testis-specific transcription of *β2-tubulin* (Kempe, Muhs et al. 1993).

Therefore, although the elements are regulated at the transcriptional level in a similar manner, the underlying sequence motifs that mediate this are not easily defined, despite the evidence that tMAC and tTAF associate with them. Indeed, efforts to determine a consensus sequence for male-germline specific transcription have been relatively inconclusive (Honeycutt and Gibson, 2004).

Comparatively little is understood regarding Medfly spermatogenesis; essentially none of the key genetic regulators have been experimentally confirmed. However, the overall process appears to be relatively similar, at least in terms of cellular organisation and the progression of divisions (Intra et al., 2011), (IAEA/FAO, 2016), despite the striking contrast in the morphology of the testes (Marchini et al., 2003). As in *D. melanogaster*, spermatogonia are associated with cysts and mitotically divide to form primary spermatocytes; there are two subsequent meiotic divisions without full individualisation of the spermatids; and finally the tails of spermatids elongate prior to individualisation and migration to the deferent duct (Shahjahan et al., 2006), (Scolari et al., 2008), (Intra et al., 2011). However, relative to *D. melanogaster*, there is one additional mitotic division that occurs prior to spermatid formation. 32 primary spermatocytes divide twice to form 128 spermatids (Intra et al., 2011). Homologues of *β2-tubulin*, the protamine-like genes *Mst35Ba* and *Mst35Bb,* and the majority of tMAC and tTAF genes are present (NCBI, 2016). The Medfly *β2-tubulin* and protamine-like homologues were used as regulators of transgenic effectors, and are discussed routinely throughout this study (**Chapters 3-5**). Alignments of the tMAC and tTAF homologues are provided in the Appendix (**Figure 8.1**). Although these genes were not investigated in this study, it would be interesting to generate mutant strains for these genes with CRISPR (clustered regularly interspersed palindromic repeats) technology (**Chapter 6**). In terms of reproductive biology, it would be interesting to experimentally confirm their role in regulating spermatogenesis. In practical terms, their regulatory elements might be useful for the targeted localisation of transgenic sterilants. Alternatively, some may provide useful targets for the development of Medfly population control strategies, for by blocking their expression by RNA interference or synthetic repressors. For instance, targeted repression of the *misfire* gene could theoretically be applied to prevent fusion of the sperm and egg, without an effect on the quantity or morphology of sperm transferred to females upon mating (Ohsako et al., 2003).

1.2.6 Genetic engineering

Expression constructs with genetically engineered effectors can be readily integrated into the Medfly genome by transposition (Gong et al., 2005). Medfly has been transformed with several transposons, including *piggyBac* (Handler et al., 1998), *minos* (Loukeris et al., 1995), and *hermes* (Michel et al., 2001). *piggyBac* vectors are easy to generate, efficiently transformed, and effective in a variety of insect species (Handler, 2002). Therefore, all transformation experiments in this thesis have utilised *piggyBac*. The native *piggyBac* sequence is an autonomous 2475 bp element that encodes the transposase required for mobilisation, within the transposon itself (Li et al., 2001a). The transposon terminates in palindromic inverted terminal repeats (ITRs), which are essential for transposition (Elick et al., 1997). The transposon can integrate via a cut-and-paste mechanism at any TTAA sequence (Li et al., 2001b). However, integration is heavily biased to particular chromosomal regions, and frequently occurs within or nearby transcribed sequences (the first intron and first exon, especially the 5'UTR, were particularly favoured) in several organisms, including human T-cells, *D. melanogaster* and *P. falciparum* (Thibault et al., 2004), (Balu et al., 2009), (Galvan et al., 2009). Furthermore, there is a significant preference for TTAA rich sequences (Balu et al., 2009), (Galvan et al., 2009). Therefore, it appears that insertion is most likely to occur in TTAA-rich, transcriptionally open chromatin. In most genetic engineering systems, the *piggyBac* transposase is removed from the native transposon and regulated by appropriate sequences for the target species in a helper plasmid (Handler and Ii, 1999). For instance, the Medfly helper plasmid applies Cchsp70-regulated *piggyBac* transposase. Thereafter, a transposable *piggyBac* vector is generated by cloning a sequence of interest between the ITRs required for transposition. Microinjection of this vector with the helper plasmid facilitates germline transformation of Medfly (Gong et al., 2005).

A typical *piggyBac* vector includes a screenable transformation marker, often a fluorescent protein, which allows transformants to be efficiently selected (**Figure 1.4**). In Medfly, the baculovirus-derived HR5IE1 promoter-enhancer or a fragment from the Mexfly muscle actin gene (comprising the promoter, 5'UTR and 3'UTR) mediate efficient fluorescent marker expression in transgenic individuals (Fu et al., 2007), (Koukidou et al., 2016). After microinjection of the *piggyBac* vector and a helper plasmid into pre-blastodermal embryos, a minority of germline cells are transformed. Subsequent backcrossing to wild-type allows the selection of stable transgenic individuals, which have the *piggyBac* integration in all cells.

An additional system for the targeted engineering of Medfly is CRISPR (**Figure 1.4**). This system was originally adapted from *S. pyogenes*, where it functions as an adaptive immune system that specifically cleaves target DNA (Brouns et al., 2008). However, a variety of CRISPR systems with alternate specificity and modes of action are known (Fonfara et al., 2014). A simplified version of the Class II CRISPR system was applied, which uses a single targeting guide RNA (sgRNA) to direct the Cas9 nuclease to a target sequence (Jinek et al., 2012), (Bassett et al., 2013). This facilitates the engineered cleavage of target sequences immediately upstream of the protospacer adjacent motif (PAM), to a variety of ends (Wiedenheft et al., 2012). For instance, genes can be mutagenised to study function (Shalem et al., 2014). Alternatively, Cas9-induced double-stranded breaks can be introduced, to then facilitate targeted recombination, for efficient sequence replacement or deletion (Saintigny, 2001). Therefore, previous transgenic lines could be engineered to express novel features, or a transgenic insertion could be directly targeted to a genomic site with a desirable expression profile. A full introduction of CRISPR systems and their functional applications is provided in Chapter 6, where the platform was validated in Medfly.

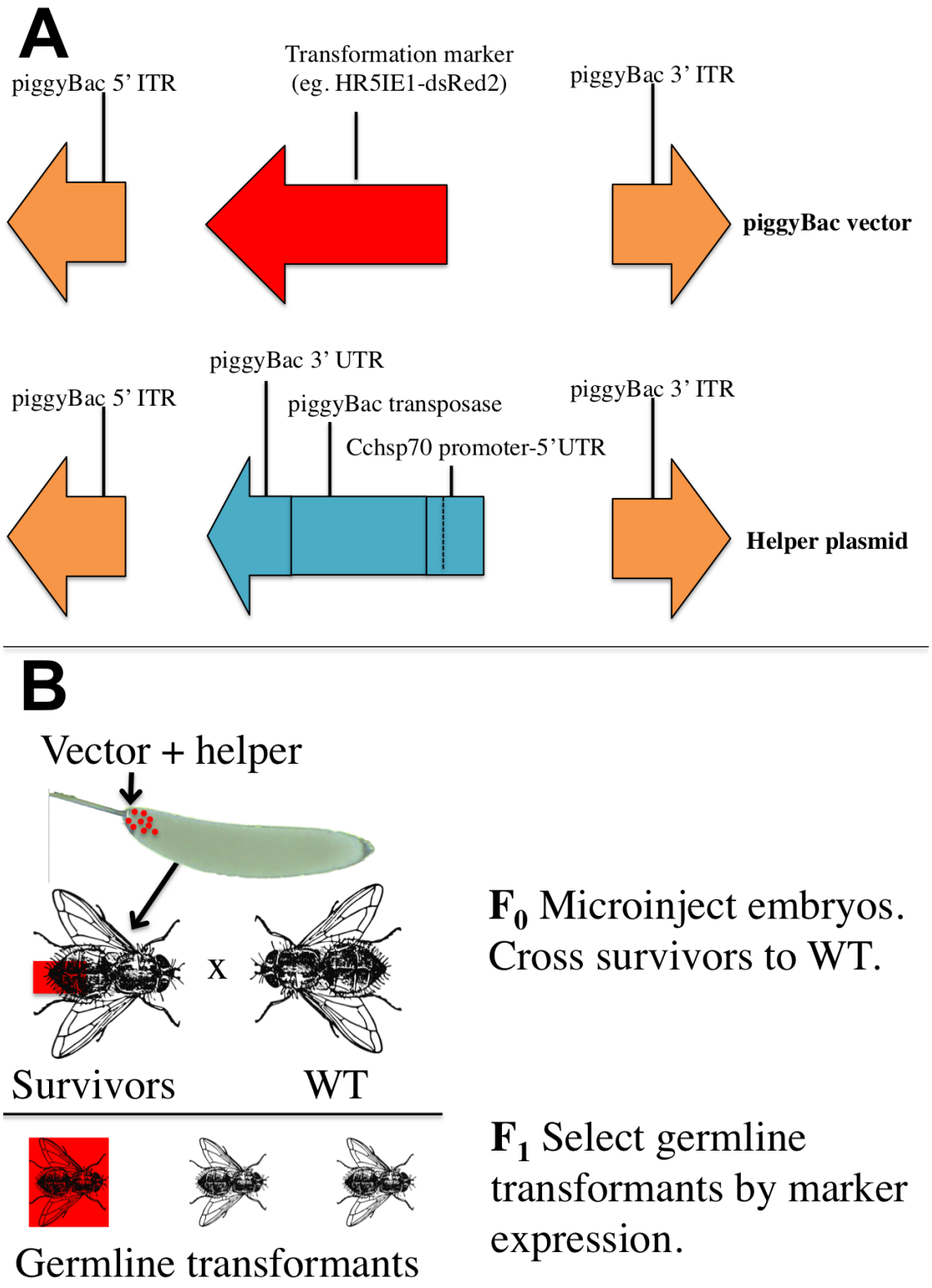


Figure 1.4. Genetic engineering in Medfly (continued on next page). (A) Stable germline transformation of Medfly can be mediated by the microinjection of a piggyBac vector containing a fluorescent transformation marker and a helper plasmid encoding transposase. (B) The process of microinjection and backcrossing to generate germline transformants. A minority of microinjection survivors will have germline cells with piggyBac vector integrations. Backcrossing these individuals to WT will allow for the selection of stable germline transformants, clonally derived from the germline cell with the integration event.

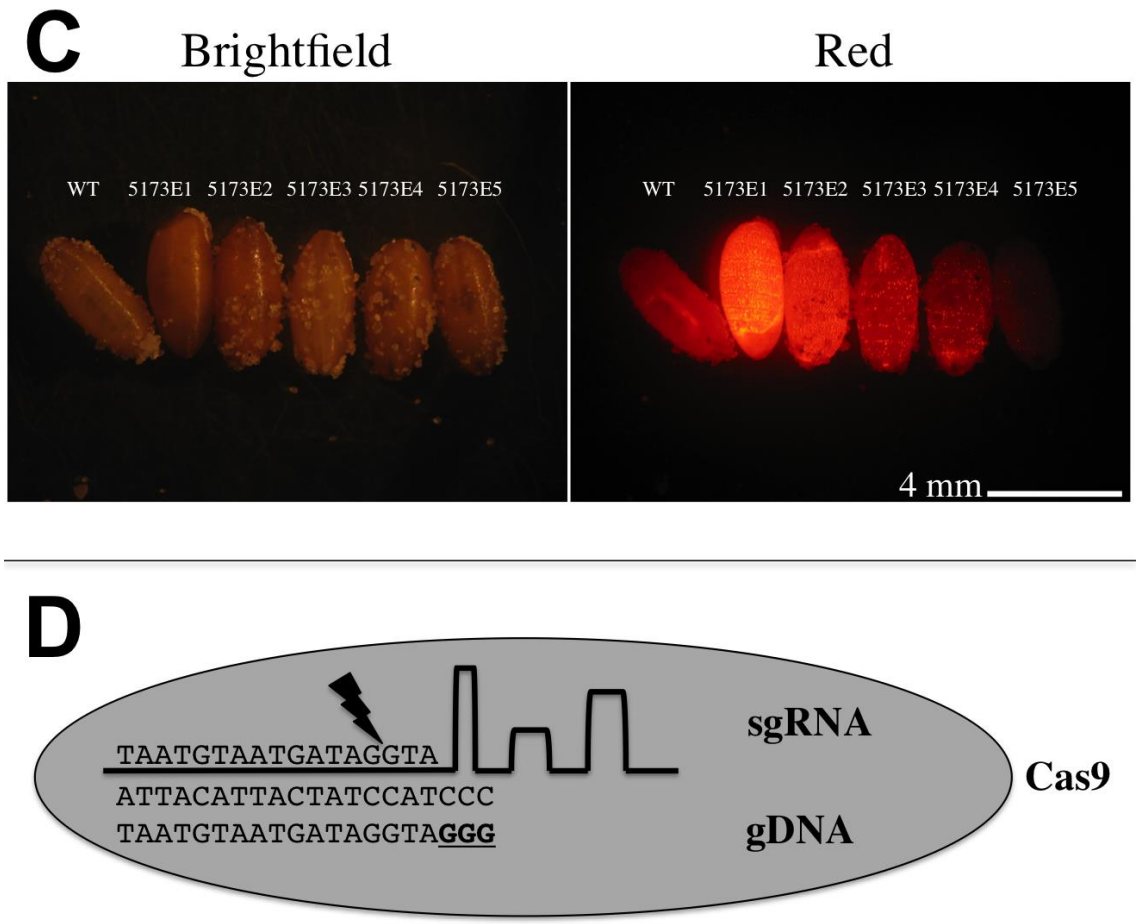


Figure 1.4. Genetic engineering in Medfly. (C) Stable germline transformants (OX5173) expressing the HR5IE1-dsRed2 marker. (D) Schematic diagram of the CRISPR system for targeted genetic engineering. The engineered sgRNA binds to a complementary genomic site, and is cleaved by the Cas9 effector near the nGG protoadjacent spacer motif (PAM). This facilitates mutagenesis or targeted sequence replacement. Embryo microinjection image adapted from Asadi (2013).

1.3 Methods for the control of pest insects

1.3.1 Preventing the introduction of pest species

The costs associated with crop loss and the control of accidentally introduced insect species are estimated at $14.4 billion, in the United States alone (Pimentel et al., 2002). The broad host range and climate tolerance of Medfly allows it to invade novel habitats, with devastating consequences (Myers et al., 2000). Therefore, vigilant monitoring is required to prevent establishment in new territories. The import of fresh fruit from infested zones to non-infested zones is carefully regulated. Several nations will not import fruit from infested areas unless processed (Jackson, 1971), (Hallman, 2000), (Mansour and Mohamad, 2004). For instance, fruit exported from Hawaii to the mainland United States is routinely irradiated, to destroy viable embryos and larvae (Follett and Lower, 2000), (Moy and Wong, 2002). Other potential treatments include heating, chilling, and fumigation (Lurie, 1998). Furthermore, routine physical and X-ray

screening checks are made in susceptible areas, to detect the presence of potentially infested fruit in passenger luggage (Liebhold et al., 2006). Further effective measures include the release of sterile male flies as a cautionary measure, to prevent the establishment of an invasive species if accidentally imported (Kuba et al., 1996). The use of sterile males to control insect populations is the central objective of this thesis, and is discussed later (**Sections 1.3.7 and 1.4**). Prophylactic measures to prevent the establishment of Medfly are of paramount importance, because in the absence of an established population, active forms of population control are not required.

1.3.2 Chemical insecticides

Chemical insecticides are substances that are toxic to any insect life stage. They can be differentiated by chemical structure, mode of action, environmental persistence, off-target toxicity and origin (natural or synthetic). Chemical control of pest insect populations emerged in the 1940s as the most common control method, following the practical success of dichlorodiphenyltrichloroethane (DDT) in controlling vectors of malaria (Casida and Quistad, 1998). Most insecticides interfere with neurotransmission (Vontas et al., 2011). The most common types are carbamates, organophosphates, organochlorines, and pyrethroids. However, an emerging class of insecticides have hormonal effects that inhibit juvenile development (Dhadialla et al., 1998), (Wu et al., 2006), (Mains et al., 2015). Naturally-derived insecticides, for instance spinosad, are promising in terms of limiting side-effects to non-target species, but they have not been widely applied (Salgado, 1998), (Isman, 2006). From each class of insecticide, the chemical structure and mode of action of a representative member is presented (**Figure 1.5**).

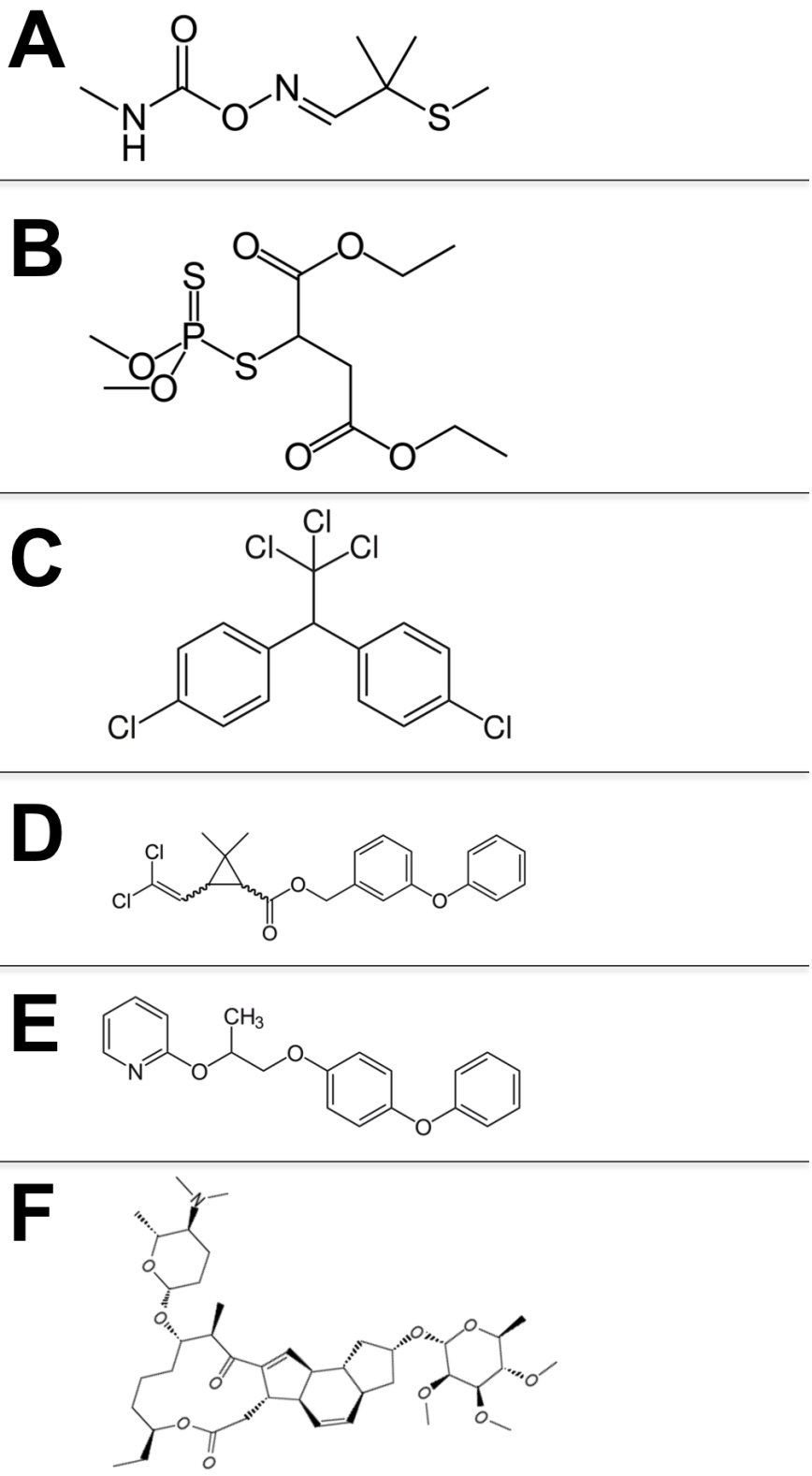


Figure 1.5. Representative examples of the chemical structures and modes of action of six common classes of chemical insecticide. (**A**) Aldicarb, a carbamate. Inhibits acetylcholinesterase; acetylcholine accumulates at synapses. (**B**) Malathion, an organophosphate. Inhibits acetylcholinesterase; acetylcholine accumulates at synapses. (**C**) DDT, an organochloride. Constiuitively activates signalling at sodium channels. (**D**) Permethrin, a pyrethroid. Constiuitively activates signalling at sodium channels. (**E**) Pyriproxyfen, a juvenille hormone analogue. Prevents larval development. (**F**) Spinosyn A, a natural insecticide derived from bacteria. Prevents nicotinic acetycholine and GABA signalling.

Though effective, the widespread application of insecticides is controversial. Many insecticidal compounds are directly toxic to non-target insect species, and may impact ecology or remove natural enemies that control pest populations (Shetty, 2004). Furthermore, they may be toxic to mammals and birds (Fukuto, 1990), (Mineau et al., 2001), (Mineau and Whiteside, 2013). They can also accumulate in the food chain (Walker, 1990), a particularly severe problem for compounds with off-target toxicity and ability to persist in the environment or biological tissue, such as dieldrin and DDT (Beyer and Krynitsky, 1989). Modern insecticides, such as neonicotionoids, are often selectively toxic to insects (Casida and Quistad, 1998), (Tomizawa and Casida, 2003). However, they rarely discriminate between pests and ecologically important insects.

Insecticide misuse selects for genetic resistance, for which three primary mechanisms are known: reduced uptake of the compound, enhanced detoxification, and mutation of the binding site (Soderlund and Bloomquist, 1990). Insecticides remain largely effective for Medfly, with the exception of malathion, for which field resistance is known (Magaña et al., 2008). However, resistance to organophosphate, dieldrin and DDT have been induced in laboratory populations of Medfly, indicating that such resistance is possible, and thus may occur in the field (Georghiou, 1986). Cross-resistance may present a further issue, because many insecticides have similar effects (Casida and Quistad, 1998), (Vontas et al., 2011). Furthermore, spraying does not effectively eradicate pests capable of long-range dispersal, like Medfly (Meats and Smallridge, 2007). Therefore it appears that insecticides are not adequate as a single solution to control Medfly, without the application of further control strategies.

1.3.3 Predator introduction

Pests are frequently controlled by competition with other insect species, or direct predation. Natural parasite wasps suppress wild populations (Yokoyama et al., 2008), (Wang et al., 2010). Carabids, *P. rufipes* and *H. distinguendus* prey upon Spanish populations of Medfly pupae (Urbaneja et al., 2006). In the Argan forest (Morocco), four species of ants were associated with about half of larval mortality under favourable temperature conditions (El Keroumi et al., 2010). In Regio Calabria (Italy), about 27% of larvae were preyed upon by ants (Campolo et al., 2015). Further studies have demonstrated that spider and ant species can effectively limit wild populations of fruit flies (Monzó et al., 2009), (Fernandes et al., 2012). It has been suggested that

intentionally introduced competitors could suppress pest species (Myers et al., 2000). However, these predators are not always species specific, and may therefore affect the population of other insects or crops (Piñol et al., 2012). Therefore, their introduction must be carefully considered to avoid disrupting the ecosystem.

1.3.4 Pheromone spraying, oviposition deterrence and trapping

Medfly respond to a variety of chemicals which alter behaviour, and therefore could potentially be used as a means of pest control (Jang et al., 1989), (Papadopoulos et al., 1998). Medfly faeces contain pheromones which discourage further oviposition to fruit; these could be applied to discourage egg-laying (Arredondo and Díaz-Fleischer, 2006). Alternatively, a pheromone deposited during oviposition is known to discourage further egg laying (Prokopy et al., 1978), (Papaj et al., 1992). Foreign chemicals, such as neem extracts, are known to possess the same oviposition deterring effect (Silva et al., 2012). Disrupting the reproductive capacity of a wild population by applying volatile compounds to discourage oviposition, would be expected to reduce the population. Despite validation of this strategy in small-scale field studies (Arredondo and Díaz-Fleischer, 2006), to our knowledge, it has not been applied on an area-wide scale to control wild Medfly populations.

A related strategy applies pheromones to disrupt the incidence of mating, which has been successful in reducing crop damage associated with the moth species *P. gossypiella* and *G. molesta* (Minks and Cardé, 1995). In Medfly, males are known to produce at least 56 volatile compounds (Jang et al., 1989). A synthetic blend of five of these compounds was effective at attracting females within a flight tunnel, though to a lesser extent than the natural emissions of live males (Jang et al., 1994). This indicates that wide dispersion of these compounds could potentially disrupt mating in wild populations of Medfly. To date, this has not been evaluated as an area-wide control strategy for Medfly, probably for practical reasons. The ability of male-derived pheromones to lure females in the field was frequently unimpressive, though it is possible that an effective formulation has yet to be described (Tan et al., 2014). Furthermore, a wide variety of plant compounds highly attractive to Medfly exist, which lack the chemical complexity of the male pheromone blend (Light et al., 1988), (Witzgall et al., 2010). Interestingly, some of these compounds (parapheromones) elicit responses similar to pheromones (Navarro-Llopis et al., 2011), and can be applied to effectively trap and kill adult populations of Medfly. Sticky traps baited with the

parapheromone Trimedlure are effective at capturing adults (Harris et al., 1971), as are protein-baited traps with pheromones (Katsoyannos, 1994). Therefore, baited traps would prove useful in an integrated pest management strategy, though further measures would be necessary to achieve full suppression.

1.3.5 Agricultural practices and engineered crops repellant to insect pests

Agricultural practices can reduce crop susceptibility. The *push-pull* strategy applies factors that push insects away from the target crop and pull them towards an alternative factor. Push factors include visual cues, repellant chemicals, alarm pheromones, and oviposition deterrents. Pull factors include visual cues, attractive chemicals or pheromones, and taste stimulants (Cook et al., 2007). A push-pull strategy suitable for Medfly would, for instance, involve discouraging oviposition with pheromones and thereafter attracting adults to traps. However, these strategies are less effective for species with a high population growth rate and wide dispersal (Cook et al., 2007), (Meats and Smallridge, 2007). Therefore, they may not be optimal to control Medfly.

An alternative strategy involves the genetic engineering of commercially important crops for pest tolerance. For instance, Roundup-Ready® soybeans express a transgene from *A. tumefaciens* conferring tolerance to glyphosphate, a common herbicide used in agriculture (Qaim and Traxler, 2005). *Bacillus* species produce a variety of crystalline toxins, some of which are highly species specific (de Maagd et al., 2003). This faciliates the development of transgenic crops that will demonstrate minimal or no toxicity to other insect species. Commercial crop strains have been developed to express a variety of these toxins, including Cry1Ab, Cry3B, Cry1F and Cry9C (Arpaia et al., 2000), (Reed and Halliday, 2001), (Nguyen and Jehle, 2007), (Buntin, 2008). Transgenic maize and cotton strains expressing these Cry proteins effectively control pests, and can improve yields and reduce costs associated with insecticide spraying (James, 2003), (Wu et al., 2008). Certain strains of *B. thuringiensis* (eg. *Bt* 13.4) are toxic to Medfly larvae and adults, though the majority are not highly effective (Vidal-Quist et al., 2009), (Aboussaid, 2010). Toxins active against Medfly have been identified from other species, for instance from *Bacillus pumilus* strain 15.1 (Molina et al., 2010). However, an engineered crop expressing an insecticide active against Medfly remains to be developed. This does not, however, preclude the direct spraying of *Bacillus*-derived insecticidal residues on crops. In some respects, direct

application would be politically favourable, as it circumvents the controversies associated with traditional insecticides and genetic engineering.

Although GM crops have been widely grown in certain countries (eg. Argentina), they remain highly controversial, particularly evidenced by protests, low approval in opinion polls, and sabotage of GM trials (Gaskell, 1999), (Rowe, 2004), (Qaim and Traxler, 2005), (Kuntz, 2012), (Mielby et al., 2013), (Hilbeck et al., 2015). The arguments against GM crops include moral concerns about interfering with nature, unanticipated effects on the ecosystem, potential risks to humans and other animals, escape of the engineered genes into the wild, lack of transparency in labelling GM foods, and the ethical tenability of patenting food (Ferber, 1999), (Gaskell, 1999), (Shaw, 2002), (Gaskell et al., 2004). A further issue is the potential for evolved resistance to GM crops, which has been observed in the field for Bt-expressing cultivars (Heckel et al., 2007), (Fabrick et al., 2014), (Gassmann et al., 2014). However, the application of GM crops is frequently associated with greater yields (Carpenter, 2010) and reduced pesticide input (James, 2003), (Finger et al., 2011). Furthermore, a lack of appreciable risk has been demonstrated for many common concerns cited against GM crops, including allergenicity to humans (Beachy et al., 2002), the risk of horizontal gene transfer (Keese, 2008), and toxicity to butterflies (Sears et al., 2001). However, approval of the platform remains low in Europe (TNS Opinion & Social, 2010). Therefore, substantial increases in public approval are required to facilitate their adoption in all regions affected by Medfly.

1.3.6 Wolbachia

Over half of insect species are estimated to be infected with Wolbachia, a group of maternally-transmitted intracellular bacteria (Hilgenboecker et al., 2008). The type species is *W. pipientis*, first isolated from the mosquito *C. pipiens* (Hertig, 1936). Four main effects are attributed to *Wolbachia* species: male-killing, feminisation, parthenogenesis and cytoplasmic incompatibility (Hurst et al., 1999). The latter is most relevant to the control of insect pests. Cytoplasmic incompatibility is a phenomenon in which the viability of a mating event is directly attributed to the infection status of the parents (Werren et al., 2008). Mating events between infected males and uninfected females lead to zygotic arrest, because chromosomes fail to partition appropriately during cellular division (Tram et al., 2006). All other combinations of mating are fertile. This enhances the transmissibility of Wolbachia, by increasing the rate of female

infection and therefore the rate of vertical transmission to progeny. This effect may be unidirectional or bidirectional: requiring infection of males and females with one or two strains, respectively (Blagrove et al., 2012).

Virulent infections of Wolbachia can reduce the ability of insect pests to survive, indicating that they can be applied to weaken wild populations (Teixeira et al., 2008), (Moreira et al., 2009), (Kambris et al., 2010). A second potential application of Wolbachia is for the direct reproductive control of pest insects (the incompatible insect technique). In this method, the cytoplasmic incompatibility of mating between infected males and uninfected females is applied to reduce field populations (Bourtzis, 2008), (Saridaki and Bourtzis, 2010). For particular strains of Wolbachia, the progeny of these mating events are completely or almost completely inviable (Blagrove et al., 2012). Therefore, the mass-release of males infected with a strain of Wolbachia absent in the wild, would be expected to severely reduce the reproductive capacity of wild insects. Strains of Medfly with Wolbachia-induced cytoplasmic incompatibility have been generated (Zabalou et al., 2004), (Zabalou et al., 2009). This strategy has also been applied in the field for mosquito control (O'Connor et al., 2012), though concerns about its biosafety and efficacy are recognised (Popovici et al., 2010).

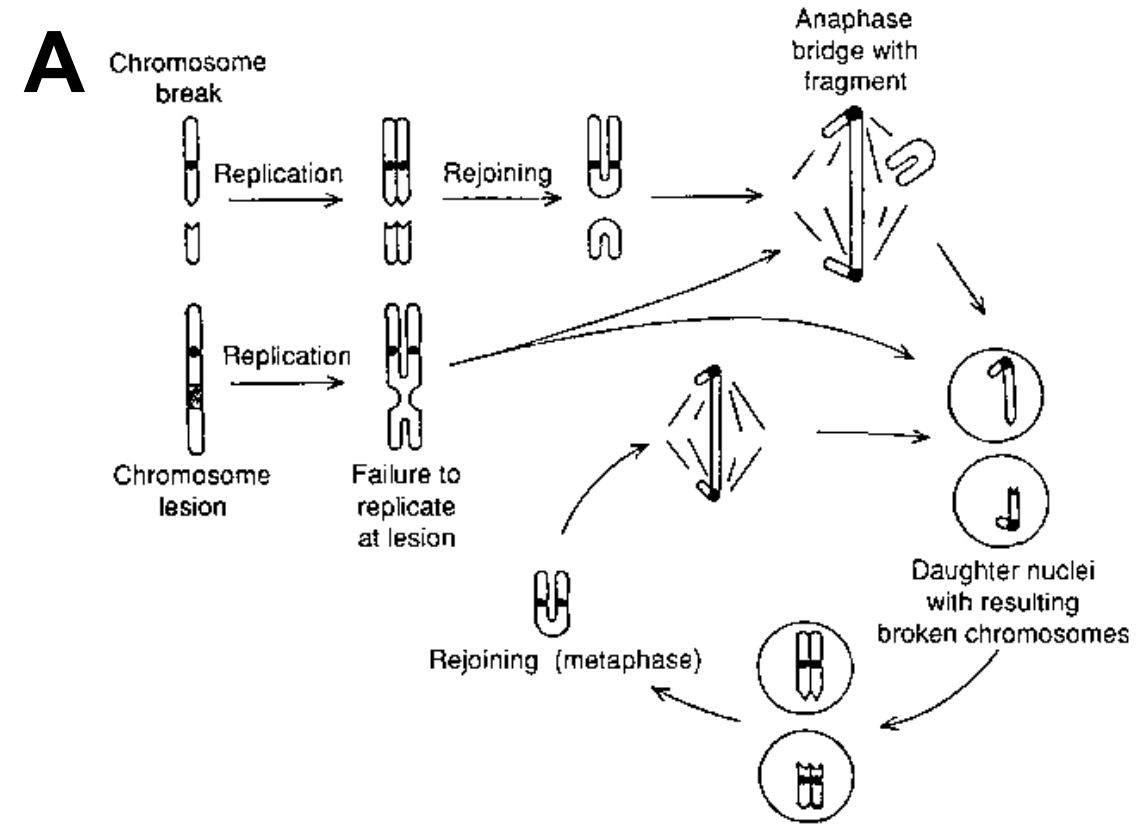
Notably, the success of this strategy is contingent upon a male-only release and complete absence of natural infection with the Wolbachia strain. If naturally infected females were present, the technique would be substantially less effective, because crosses of infected males and infected females are fertile. Similarly, the release of a single Wolbachia infected female from rearing facilities would allow the infection to potentially sweep through the population (by vertical transmission) and would therefore block cytoplasmic incompatibility. The sex-separation strategies employed in prior wild releases of Wolbachia-infected males were imperfect, and it has been conceded that release of infected females would be expected at low frequency with current non-genetic separation techniques (Balestrino et al., 2014), (Calvitti et al., 2015). This could be avoided by the application of highly effective genetic sex-separating strains to yield male only populations, but such strains would need to be 100% effective (Zabalou et al., 2009).

Another concern associated with Wolbachia technologies is lateral gene transfer, which can occur between the host and the introduced Wolbachia species. This has resulted in the transfer of fragments ranging from gene-sized cassettes to nearly-

complete Wolbachia genomes (Kondo et al., 2002), (Hotopp et al., 2007), (Hou et al., 2014). This could potentially permanently alter the genetic composition of wild species with unknown effects, posing a substantial ethical concern. A further issue concerns the wild release of Wolbachia into the environment, which is not easily recalled; novel infections can sweep through the wild population in less than a decade (Kriesner et al., 2013). Therefore, the strategy may prove effective in future application, if several conditions are met: releases of infected insects are exclusively male; the effect of Wolbachia infection on vectorial capacity is fully understood; and the penetrance of cytoplasmic compatibility is complete. However, as cytoplasmic incompatibility is rarely entirely penetrant, the strategy must be approached with utmost caution (Zabalou et al., 2004) .

1.3.7 The sterile insect technique (SIT)

The SIT (**Figure 1.6**) is a strategy for the chemical-free reproductive control of pest insect species (Knipling, 1955). The population control strategy devised in this thesis is a modification of SIT, to engineer improved male competitiveness and enhanced field traceability. In the SIT, reproduction of wild pests is inhibited by the mass release of sterilised males, which dilutes the reproductive ability of the population (Baumhover et al., 1959), (Takken, 1987), (Alphey, 2006). Prolonged release of sterile males in sufficient number has substantially reduced or eradicated wild populations. Successful programmes have been applied for several species and several are ongoing (**Table 1.1**). The success of the strategy is dependent upon several factors: reliable male sterilisation that minimally affects competitiveness, reliable sex separation to yield male-only release populations, appropriate scale of release, and diligent monitoring in the field (Alphey, 2002), (Rendón et al., 2004), (Black et al., 2011).



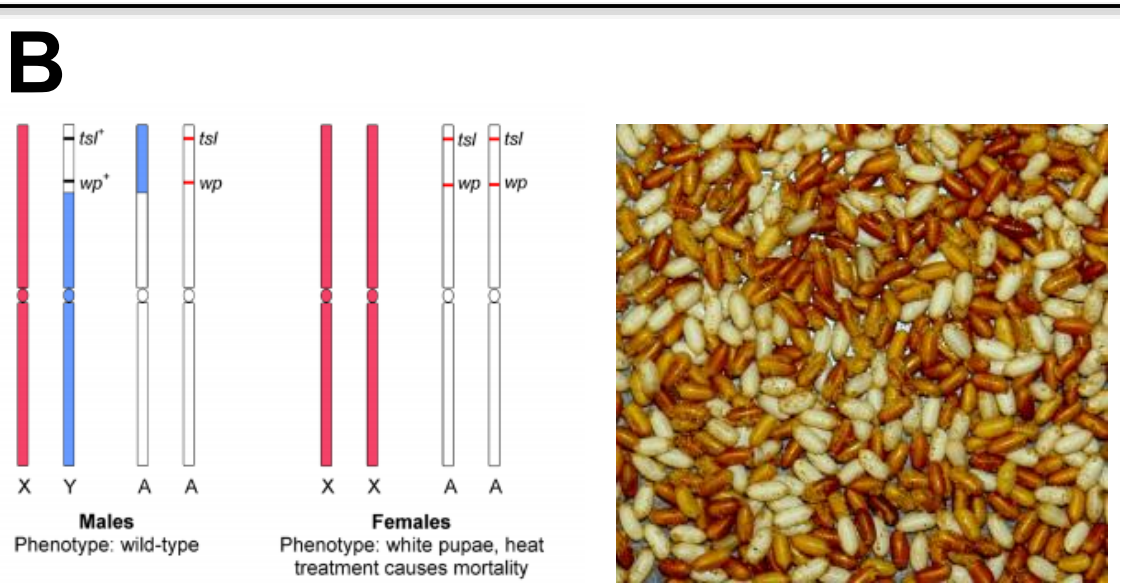


Figure 1.6. The sterile insect technique (continued on next page). (A) Irradiation sterilises males by introducing dominant lethal mutations into sperm. This leads to chromosomal aberrations, preventing the formation of viable zygotes when mated to wild females. (B) The temperature-sensitive lethal (tsl) VIENNA-8 genetic sexing strain, commonly applied in SIT programmes. Left: A translocation provides male-specific linkage of the selectable tsl trait and a visual marker. Right: Male (brown) and female (white) pupae.

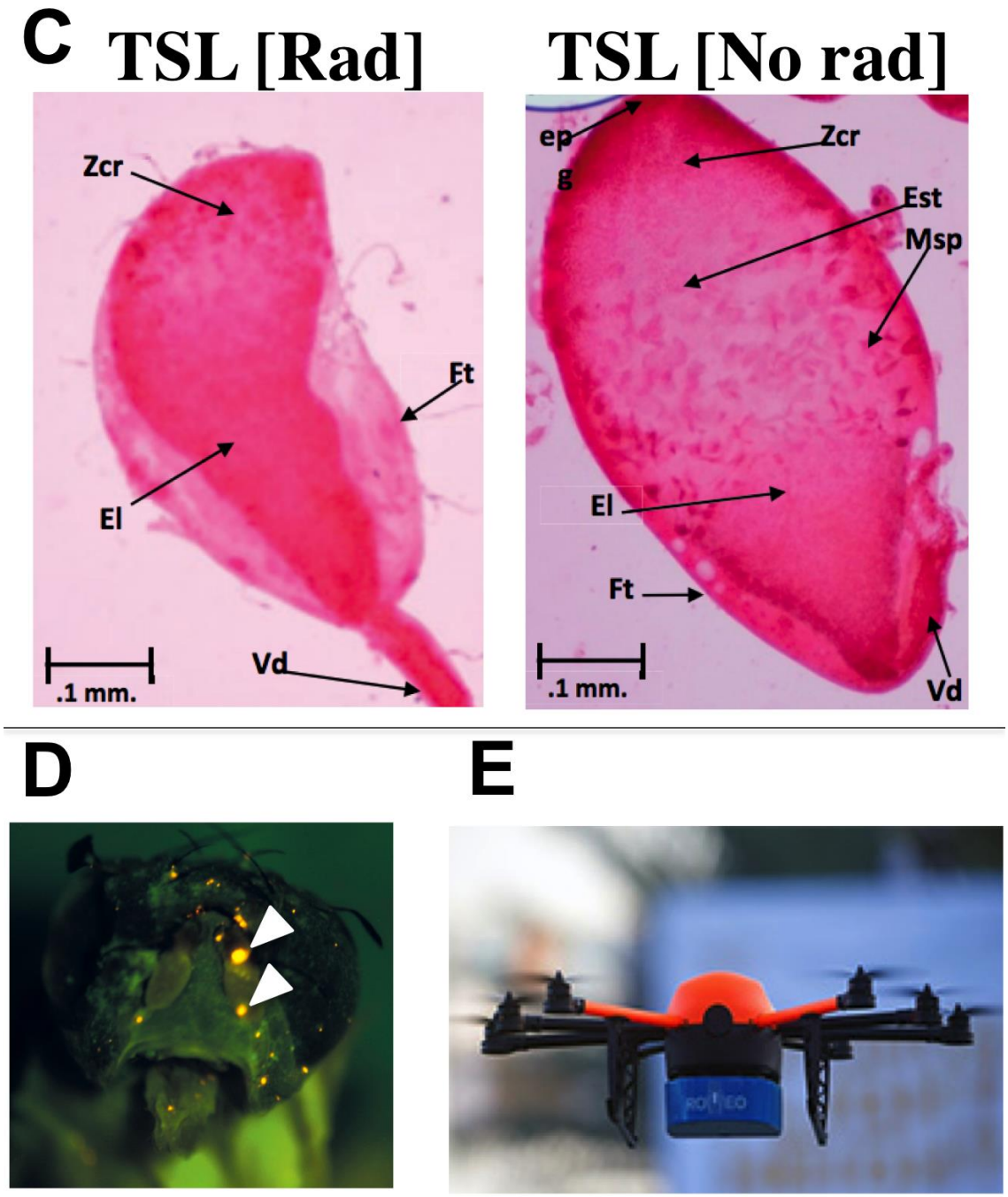


Figure 1.6. The sterile insect technique. (C) Effects of irradiation on testes of tsl males at 10 days. Left: irradiated (145 Gy); Right: not irradiated. Abbreviations: El = Free sperm; epg = Spermatogonia; Est = Spermatids; Ft = Testicular sheath; Msp = Sperm bundles; Vd = Vas deferens; Zcr = Growth area. (D) Dusting males with a traceable fluorescent powder (indicated by white arrowheads). (E) Mass-release of sterile males from a drone. Image credits: A: FAO (http://www.fao.org/docrep/u4220t/u4220T0j.htm); B: Morrison *et al* (2010); C-D: IAEA/FAO (2016); E: IAEA (http://www-naweb.iaea.org/nafa/ipc/drones-romeo-mosquitoes.html)

Table 1.1: Partial list of previous and ongoing SIT programmes

Species	Region	Date	Outcome	Notes	Ref
C. hominivorax	USA & Mexico	1956-1991	Eradication		(Wyss, 2006)
	Guatemala & Belize	1986-1994	Eradication		(Wyss and Galvin, 1996)
	Libya	1990-1991	Eradication		(Lindquist et al., 1993)
	El Salvador	1991-1995	Eradication		(Galvin and Wyss, 1996)
G. austeni	Unguja, Zanzibar	1994-1997	Eradication		(Vreysen et al., 2000)
G. p. palpalis	Central Nigeria	1986	Eradication		(Takken et al., 1986)
Medfly	Western Australia	1978-present	Suppression	Eradication not achieved, sterile releases continue as a preventative measure	(Department of Agriculture and Food, Western Australia, 2015)
	California, USA	1980s-present	Suppression	Routine outbreaks since 1980s, preventative releases of sterile males continued	(Jackson and Lee, 1985), (Headrick and Goeden, 1996)
	Guatemala	1983-present	Suppression	Suppression achieved, preventative releases continued	(Linares and Valenzuela, 1993), (Moscamed, 2016)
	Chile	1988-1995	Eradication		(Lobos and Machuca, 1998)
	Argentina	1996-present	Suppression	Preventative releases to reduce population	(De Longo et al., 2000)
	Israel	1997-present	Suppression		(Rossler et al., 2000), (IAEA, 2016)
	South Africa	1997 - present		No literature available regarding efficacy	(IAEA, 2016)
Melon Fly	Okinawa, Japan	1972-1993	Eradication	Preventative sterile releases continue	(Kuba et al., 1996)
Sweet potato weevil	Japan	1999-2002	Suppressed		(Kohama et al., 2003)

Mass-released sterile males must remain competitive and able to engage in the normal mating behavior of the target species, because they must displace the mating attempts of fertile, wild males (Alphey, 2006). The mode of sterility is ideally paternal effect embryo lethal, because Medfly primarily damage crops in the larval stage (Wimmer, 2005). Currently, almost all mass-release programmes sterilise males by irradiation (IAEA, 2016). This reliably introduces dominant lethal mutations into sperm (Bakri et al., 2005), but undesirably reduces male performance in the field (Shelly et al., 1994), (Alphey, 2006). For instance, ability to produce sperm is reduced after irradiation (IAEA/FAO, 2016). Furthermore, the mating competitiveness of males is reduced by mass-rearing and sterilisation, which reduces their ability to court females and thereby reduce the population (Shelly et al., 1994), (Shelly and Whittier, 1996), (Lance et al., 2000), (McInnis et al., 2002). Poor competitiveness has been implicated in the failure of certain SIT programmes, including attempts to control *A. gambiae* in Burkina Faso and *C. tritaeniorhynchus* in Pakistan (Benedict, 2003). This can be attenuated by releasing more males, though this incurs a greater economic cost (Barry et al., 2003), and it is expected that severe deficits in male competitiveness would require an unfeasible scale of release. However, it should be noted that if the wild population is suppressed, even at sub-optimal levels, then the scale of release required to achieve suppression will be gradually reduced, as the wild population declines.

To further complicate matters, female fruit flies have complex behavioural responses to mating. Males must be appropriately sized and nourished to optimally attract females (Taylor and Yuval, 1999). Furthermore, males must successfully engage in leks; this ability is reduced in mass-reared males (Liimatainen et al., 1997), (Briceño and Eberhard, 1998), (Lance et al., 2000). Females also respond to cues in seminal fluid and to the presence of sperm in the spermathecae (Mossinson and Yuval, 2003), (Gomulski et al., 2012). It has been demonstrated that sterilisation by irradiation reduces the ability of Medfly males to induce female refractoriness to re-mating (Kraaijeveld and Chapman, 2004). Irradiation can reduce sperm count (Seo et al., 1990), which is concerning because females that receive less sperm have been shown to re-mate at a higher frequency (Mossinson and Yuval, 2003). Therefore, to effectively mediate the desired population suppression, the sperm of sterile males should be incapable of allowing development of the zygote, but otherwise equivalent to wild-type.

The requirement for male-only releases is multifactorial. Sterile females can still sting fruit, reducing its value. Mating behaviour is also a primary consideration. Males mate as often as possible; females frequently mate more than once, but are more conservative in their choice of mate (Bonizzoni et al., 2002). Consequently, releasing exclusively sterile males achieves greater suppression, as they will attempt to mate with several wild females. Field studies demonstrated that mixed sex sterile releases are less effective than male-only releases (Rendón et al., 2004). This is because mating events between sterile males and sterile females would not reduce the wild population, and would expend resources of sterile males that could be used to court wild females. To achieve a male-only population, several sex-separation strategies exist. Manual or mechanical separation is possible, but error-prone and expensive (Klassen and Curtis, 2005). For this reason, genetic strategies developed to select against females, have vastly improved the practicality of SIT. For instance, male-linked translocations of an insecticide resistance allele have been applied (Lines and Curtis, 1985). However, the application of insecticide could potentially introduce fitness penalties even at sub-lethal doses; it is also difficult to justify the release of genes conferring insecticide resistance into the field.

The VIENNA-8 temperature sensitive lethal (tsl) genetic sexing strain (GSS) was a major innovation. In this line, a translocation between the Y and fifth chromosomes provides male-specific linkage of the wild-type alleles for a selectable trait (temperature specific lethality) and a visual marker (*white pupae*; males have the normal brown colour). Females are homozygous for the recessive loss-of-function alleles. Therefore, they can be removed by heat treatment at the restrictive temperature and are visually distinguishable from males by white colour; a male-only population is derived with 99.9% accuracy (Caceres, 2002), (Franz, 2005), (Morrison et al., 2010). However, there are practical issues. During mass rearing, the genetic sexing mechanism can be disabled by rare recombination events, removing its ability to provide a male-only population (Kerremans and Franz, 1995), (Caceres, 2002). Although this risk has been substantially addressed by periodically re-establishing mass-rearing colonies from small, independently maintained filter colonies, it would be preferable to develop a sexing mechanism that was more stable (Robinson et al., 2002), (Shelly, 2012). Such a transgenic line has been developed at Oxitec (OX3097/OX3864), and is discussed later (Fu et al., 2007), (Leftwich et al., 2014).

Additionally, it is extremely important to monitor the competitiveness of released males, and to understand the reproductive biology and distribution of the wild population, as a successful strategy will require that sterile males mate wild females at an acceptable frequency (Knipling et al., 1968), (Calkins and Ashley, 1989), (Dyck et al., 2005). Field data suggests that SIT programmes in Medfly have been affected by poor competitiveness; in trials in Guatemala and Hawaii, releases of males in hundredfold excess of the wild population failed to achieve a net egg sterility target of 77% (Shelly and McInnis, 2016). Species with complex courtship, such as Medfly, may require higher release ratios, because the likelihood of rejection is greater (Shelly and McInnis, 2016). The scale of sterile male release and its effect on the population can be performed by comparing the ratio of released to wild insects on traps (Hendrichs et al., 1995), (Rendón et al., 2004). Furthermore, the mating ability of sterile males in the field can be assessed, by scoring the female reproductive tract for the presence of wild-type sperm, as well as irradiated sperm or transgenically marked sperm (McInnis, 1993), (Zimowska et al., 2009).

1.4 The Oxitec strategy is a male sterility platform improved by genetic engineering

1.4.1 Summary of the method

This study is primarily concerned with devising an enhanced strategy for the reproductive control of Medfly, by addressing the fundamental issues with traditional, radiation-based SIT (Alphey, 2006). The requirements to effectively control pest populations with sterile male release, as described in the previous section, are well understood. However, they have proven difficult to achieve in practice, primarily due to the negative effects of irradiation, laboratory colonisation, and mass-rearing on male fitness. In certain respects, the Oxitec solution is similar to traditional SIT, because it inhibits male fertility (Ant et al., 2012). However, the primary difference is the extent of targeting. Tightly regulated transgenic effectors mediate sterility, potentially avoiding the substantial off-target effects of radiation (Black et al., 2011).

We describe the development of an engineered platform to control Medfly, with repressible male sterility mediated by nuclease expression in spermatids and sperm (Jin, 2011). This is expected to reduce non-specific effects upon male fitness, by limiting translation of the sterilising effector to male germline tissue. To facilitate effective mass-rearing, efficient female removal is engineered by genetic sexing (Fu et al., 2007). Finally, field traceability is engineered by expression of fluorescent markers expressed in the body and sperm (Scolari et al., 2008). The initial characterisation of most of these components was performed by staff at Oxitec and three prior PhD students (Jin, 2011), (Bilski, 2012), (Asadi, 2013).

1.4.2 Male sterility by repressible nuclease expression (tetO-protamine-FokI)

1.4.2.1 Requirements of the system

As previously discussed, the male sterility system must be paternal effect embryo lethal, to prevent fruit destruction by hatching larvae (Wimmer, 2005). Furthermore, expression of the sterility effector in males should remove the viability of all progeny, to ensure effective suppression of the population, and prevent vertical transmission of the transgene to the wild population. Selective expression of the nuclease effector would ideally allow males to retain high mating competitiveness and

the ability to transfer morphologically normal (but sterile) sperm and seminal compounds to females during mating.

1.4.2.2 Protamine-FokI is a male sterility effector that cleaves sperm DNA

The male sterility effector is a chimeric DNA endonuclease (**Figure 1.7**), a protamine-like protein fused to the non-sequence specific DNA cleavage domain of FokI nuclease (protamine-FokI). This facilitates DNA binding and cleavage in spermatids and sperm (Asadi, 2013). The function of both components is discussed below. Protamine family genes are small, basic, positively-charged proteins that tightly package sperm DNA in a manner comparable to, but structurally very different to histones (Queralt et al., 1995), (Braun, 2001), (Balhorn, 2007). In *Drosophila*, the genes are termed protamine-like because they have a different evolutionary origin, demonstrating a clear homology to somatic histone H1 (Eirin-Lopez, 2006), (White-Cooper, Doggett & Ellis, 2008). They are transcribed in primary spermatocytes but translated in spermatids, and the proteins are components of mature sperm (Barckmann et al., 2013). During spermiogenesis, histones are removed from spermatid DNA and replaced by protamine-like proteins, which bind DNA through positively-charged domains (Jayaramaiah Raja and Renkawitz-Pohl, 2005). Protamine-DNA complexes associate with one another by the formation of disulphide bonds between cysteine residues of adjacent protamines, facilitating a greater extent of DNA compaction compared to histones (Balhorn, 2007). Three protamine-like genes have been identified in *D. melanogaster*: *Mst35Ba* (Dmprot1), *Mst35Bb* (Dmprot2) and *Mst77F*. Curiously, Mst77F is essential for male fertility, but Mst35Ba and Bb mutants are partially fertile (White-Cooper, Doggett & Ellis, 2008), (Tirmarche et al., 2014). In *Drosophila*, Mst77F is thought to bind DNA by electrostatic interaction, without sequence specificity. Thereafter, multimerisation of several DNA-Mst77F complexes tightly compacts DNA (Kost et al., 2015). Two protamine-like genes (Ccprot1 and Ccprot2) were identified in Medfly by homology searching (**Section 4.2**). Dmprot1, Dmprot2, Ccprot1 and Ccprot2 were applied in this study.

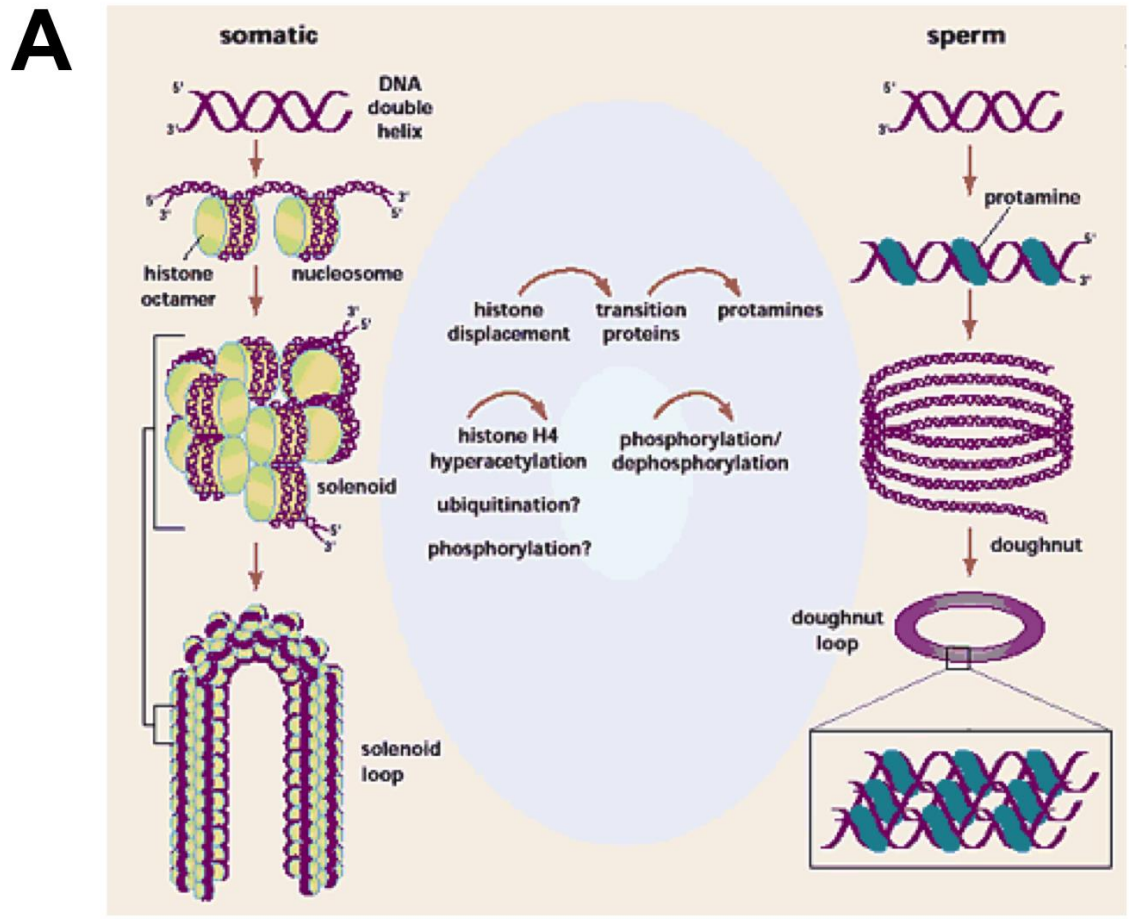


Figure 1.7. Male sterility mediated by protamine-FokI induced double-stranded breaks (continued on next page). (**A-B**) Protamine packages DNA in sperm. (**A**) Packaging of somatic and sperm DNA with histones and protamines. (**B**) Fluorescence microscopy of Mst35Bb (Dmprot2)-EGFP translation in testes. Spermatids indicated with white arrowheads.

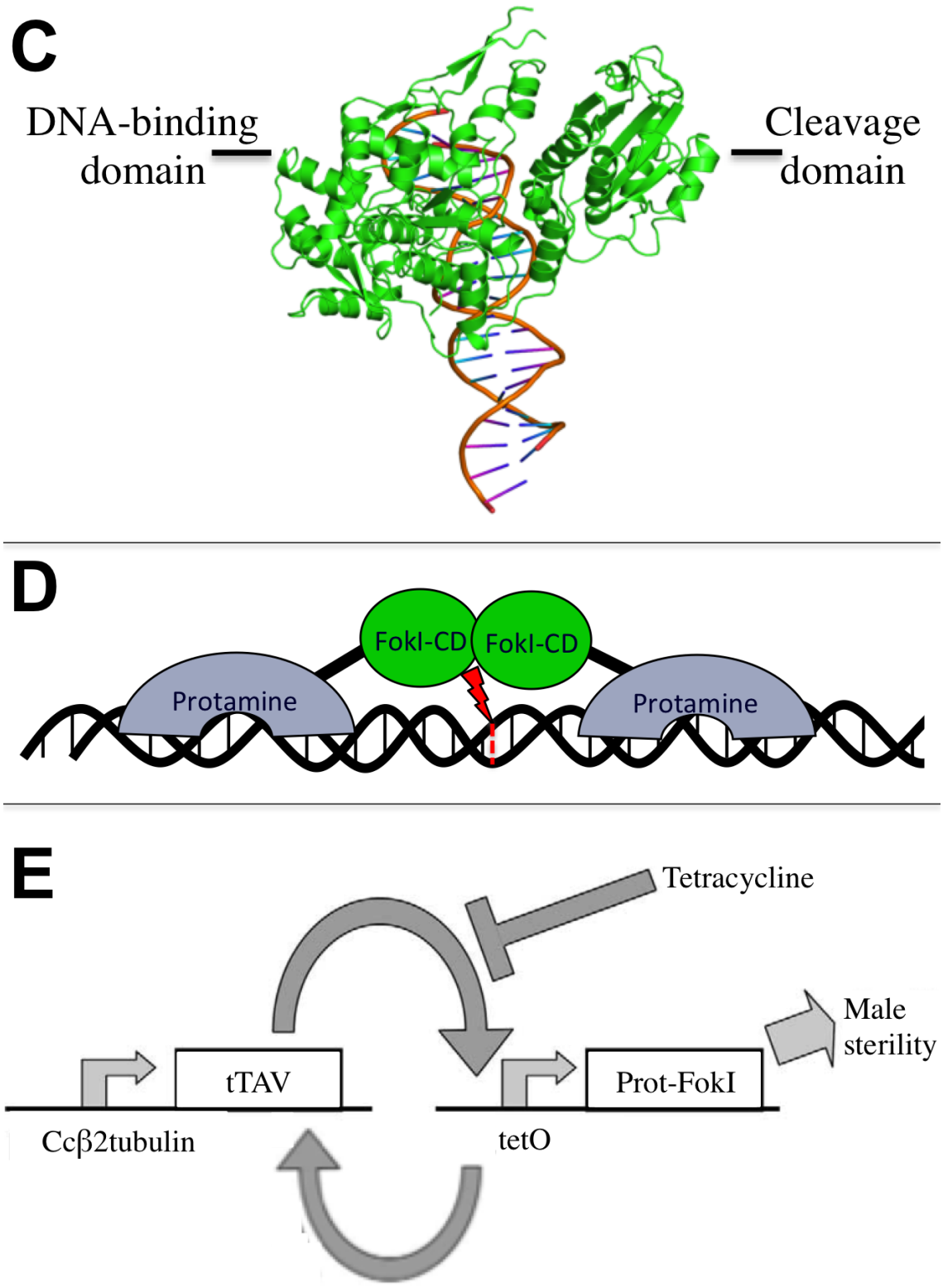


Figure 1.7. Male sterility mediated by protamine-FokI induced double-stranded breaks. (**C**) Structure of the FokI restriction enzyme bound to DNA. (**D**) The protamine-FokI cleavage domain fusion induces double-stranded breaks in sperm DNA when adjacent molecules dimerise tail-to-tail. (**E**) Expression of the tetracycline-repressible transcriptional activator tTAV with a male-germline specific promoter, allows repressible expression of protamine-FokI in sperm. Image credits: A: Braun (2001); B: Jayaramaiah Raja & Renkawitz-Pohl (2005); C: PDB; D: Adapted from Helen White-Cooper. E: Adapted from Alphey (2002).

FokI is a restriction enzyme isolated from *Flavobacterium okeanokoites* (Li et al., 1992). It is a type IIS restriction enzyme, cleaving DNA at 9-13 nucleotides from the asymmetric recognition site GGATG (Pingoud and Jeltsch, 2001). The structure of FokI is unusual, with independent domains for DNA recognition and cleavage (Wah et al., 1997). This property has made FokI incredibly useful for genetic engineering, because the cleavage domain does not demonstrate sequence specificity (Chandrasegaran, 1996). Therefore, chimeric restriction enzymes with novel specificity can be generated, by fusing a DNA-binding element to the non-specific FokI cleavage domain, as we have done with protamine-FokI (Smith et al., 1999), (Gaj et al., 2013). The native enzyme exists as a monomer in solution, but magnesium-dependent dimerisation of two adjacent FokI molecules is required to cut DNA (Vanamee et al., 2001). In the unbound state, associations between the two domains prevent the cutting domain from engaging in non-specific activity (Wah et al., 1998). However, DNA binding induces a conformational change that frees the cutting domain, allowing it to dimerise with an adjacent FokI molecule and cleave target DNA. Because we removed the N-terminal recognition domain in the chimeric protamine-FokI enzyme, this conformational change is not expected to occur.

1.4.2.3 tetO-protamine-FokI is repressibly regulated by Ccβ2tubulin-tTAV in the male germline

To minimise off-target effects, protamine-FokI should be expressed exclusively in the male germline. This is mediated by a two-part expression system (Jin, 2011), (Alphey, 2015). The first component is a tetracycline-repressible transcriptional activator (tTAV) transcribed and translated in the male germline (**tetO**-Ccβ2tubulin promoter-5'UTR[short]-Cchsp83 minipromoter-Cchsp83 5'UTR-**tTAV**-SV40 3'UTR). In the absence of tetracycline, tTAV protein accumulates in the primary spermatocytes of individuals with this expression construct. Thereafter, tTAV, bound to its transcriptional target site tetO, activates transcription of a protamine-FokI fusion protein. Additionally, tTAV is expected to increase its own expression because the tetO sequence is bi-directionally active, and located between the two transcriptional units, which are cloned in head-to-head orientation. In the presence of tetracycline, there should be minimal or no activation of expression of the tTAV target genes (Gossen, 1992), (Schönig et al., 2011). The expression of these two components (tTAV and tetO-protamine-FokI) within a single *piggyBac* vector (OX4353**)** has previously been shown to mediate penetrant and repressible male sterility in olive fly and Medfly (Jin, 2011),

(Asadi, 2013). However, leaky expression has been demonstrated: both on-tetracycline and in somatic tissue, for at least some strains (Asadi, 2013). Therefore, it is essential to assess transgenic strains for leakiness and investigate the potential effects on competitiveness or longevity.

Importantly, this system provides a mechanism to repress the engineered male sterility, allowing the strain to be propagated in the lab (Alphey, 2002). Furthermore, the requirement of tetracycline for fertility prevents both the accidental release of non-sterile males, as well as the persistence of transgenes in the wild (Alphey et al., 2008). However, engineering these features has proven difficult because of the unusual dynamics of gene expression in the male germline. Although primary spermatocytes are highly transcriptionally active, there is very little post-meiotic transcription in *D. melanogaster* (Barreau et al., 2008), (Vibranovski et al., 2010). Therefore, genes that are required for post-meiotic development in the male germline are stored as mRNAs and translationally repressed, often by elements within the 5'UTR (Kempe et al., 1993), (Jayaramaiah Raja and Renkawitz-Pohl, 2005), (White-Cooper, 2009), (Barckmann et al., 2013).

Adequate pre-meiotic translation of tTAV is therefore required to activate transcription of protamine-FokI to sufficient levels, in advance of the transcriptional arrest. Although early transcription of protamine-FokI is desirable, it should be noted that early translation is not. The protamine-FokI transcript must therefore incorporate appropriate signals for translational delay. If this is not achieved, translation of the male sterility effector will not occur in all spermatids at the appropriate time. If translation of protamine-FokI is too late, sperm are likely to retain an intact genome, and will therefore remain fertile. In contrast, early translation of protamine-FokI would be anticipated to disrupt the meiotic divisions, or cause significant defects in spermatid nuclear shaping, leading to failure of spermatid individualisation. Either outcome would be extremely problematic, because the primary objective of this study is to engineer sterile sperm that are capable of fertilisation, thereby inducing a paternal effect lethal phenotype. This is expected to improve upon radiation-based SIT, for which negative impacts on sperm morphology and number have been demonstrated, in certain instances (McInnis, 1993), (Helinski and Knols, 2009).

1.4.2.4 Development of the two-component system for repressible male sterility

A functioning system for the repressible expression of the male sterility effector was described prior to the initiation of this study (Jin, 2011). Substantial experimentation was required, because of the difficulty of engineering appropriate transcription and translation of transgenic effectors in the male germline (**Figure 1.8**). The most important experiments are summarised here.

The tetracycline-repressible transcriptional activator was developed first, primarily from components of Ccβ2tubulin and Cchsp83 (**Figure 1.8**). β2tubulin is a highly conserved gene that is transcribed specifically in the testes of a variety of insect species (Michiels et al., 1989), (White-Cooper, Doggett & Ellis, 2008), (Nielsen et al., 2010). In *D. melanogaster*, a 14 bp promoter element (β2UE1) is required for testis-specific transcription (Michiels et al., 1989). Two additional downstream elements, one within the promoter (β2UE2) and one within the 5'UTR (β2DE1), enhance expression but are dispensible for testis-specific expression (Michiels et al., 1993), (Santel, 2000). Sequence motifs with homology to β2UE1 and β2UE2 were computationally identified in the Medfly homologue (Ccβ2tubulin), but in this instance, they are present in the putative 5'UTR (Scolari et al., 2008). It is notable that their functionality in Medfly is inferred by homology, and not yet experimentally confirmed. It was anticipated that the Ccβ2tubulin promoter-5'UTR fragment could mediate early transcription and translation of tTAV, suitable for activating early transcription of tetO-protamine-FokI.

A fragment based on the Ccβ2tubulin promoter, Ccβ2tubulin 5'UTR and SV40 3'UTR (**Figure 1.8**) was able to facilitate pre-meiotic transcription and translation of a dsRed2 reporter, in spermatocytes (OX3671: Ccβ2tubulin promoter-Ccβ2tubulin 5'UTR-**dsRed2**-SV40 3'UTR). However, visible reporter translation was not observed in all spermatocytes, indicating that the system would not mediate sufficiently early translation of tTAV (Jin, 2011). To solve this issue, 233 bp of the Ccβ2tubulin 5'UTR were truncated, to remove putative signals within the 5'UTR that delayed translation. The resulting Ccβ2tubulin promoter-Ccβ2tubulin 5'UTR[short] fragment (1030 bp → 797 bp) retained the majority of the β2UE1 element expected to confer testis-specific transcription, but lost the β2UE2 element (Scolari et al., 2008), (Jin, 2011).

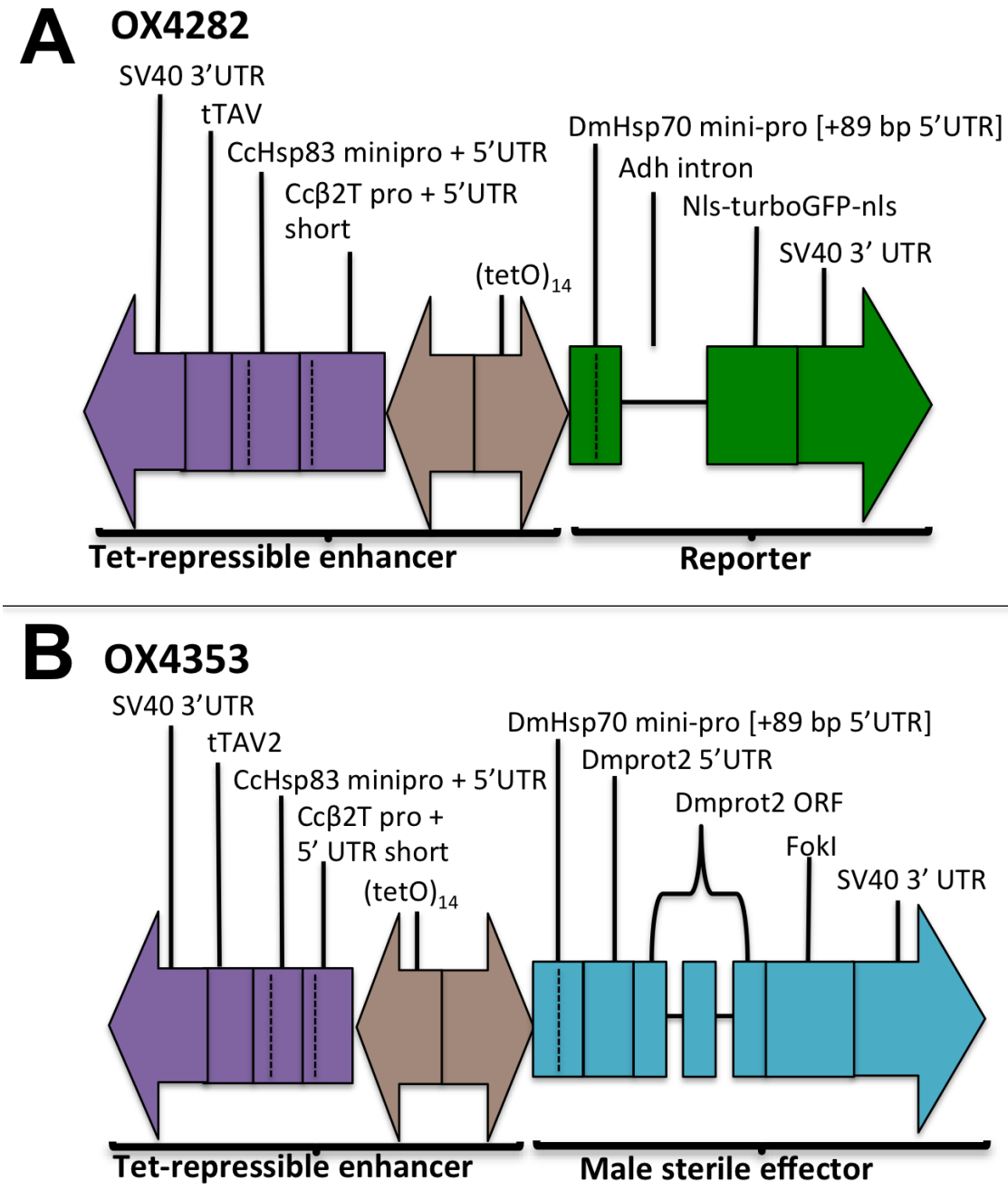


Figure 1.8. Development of the tetO-protamine-FokI system to mediate repressible male sterility (continued on the next page). (A-B) Validation of a suitable expression system for tTAV, to engineer repressible male sterility in Medfly. (**A**) OX4282 applied a truncated Ccβ2tubulin 5'UTR with a chimeric Cchsp83 minipromoter-5'UTR, to enhance early translation. (**B**-**C**) This Ccβ2tubulin-hsp83 fragment mediated penetrant-repressible male sterility by activation of a tetO-protamine-FokI effector. (**B**) OX4353 diagram.

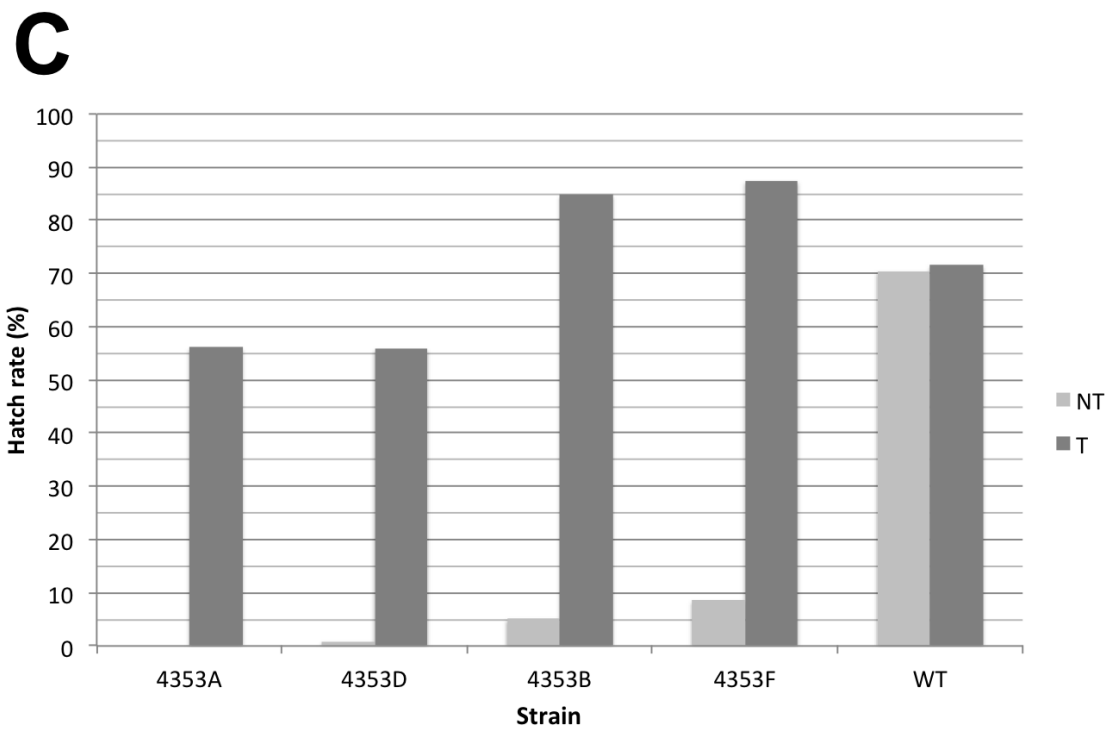


Figure 1.8. Development of the tetO-protamine-FokI system to mediate repressible male sterility. (**C**) Penetrant-repressible male sterility was confirmed by a reduced hatch rate in males reared off-tetracycline in several OX4353 strains (this assessment was performed by Romisa Asadi [Asadi, 2013].

To enhance early expression, a minimal promoter fragment and 5'UTR from Cchsp83 (a gene with earlier transcription and translation), was added downstream of the Ccβ2tubulin promoter-Ccβ2tubulin 5'UTR fragment (Ding et al., 1993). This chimeric fragment was found to facilitate appropriately early transcription and translation of a fluorescent reporter in spermatocytes (Jin, 2011). Subsequently, the chimeric Ccβ2tubulin-Cchsp83 driver was able to appropriately promote expression of tTAV, and this was able to activate a tetO-driven Dmprot2-FokI effector in the male germline (OX4353: **tetO14**-Dmhsp70 minipromoter-Dmprot2-FokI). This resulted in penetrant, repressible male sterility (Jin, 2011), (Asadi, 2013). Therefore, it appeared that the truncation of Ccβ2tubulin 5'UTR and addition of Cchsp83 minipromoter-Cchsp83 5'UTR had the desired effect of both increasing transcription level and enhancing early translation of tTAV.

1.4.3 Genetic removal of females facilitates a male-only release

A functional protamine-FokI male sterility system would be combined with a previously described tetracycline-repressible female-lethal system (OX3864, **Figure 1.9**), which serves as an effective Medfly sexing strain (Leftwich et al., 2014). This system applies an autoregulatory, positive feedback loop to fatally overexpress tTAV in females alone (Fu et al., 2007). This specificity is engineered by the inclusion of introns from *tra*, which are spliced to remove a stop codon in females, but not males (Pane et al., 2002). Therefore, female-specific expression of tTAV may be engineered by inclusion of female-specific *tra* introns within the coding sequence. Thereafter, tTAV positively regulates its own expression at an array of tetO operator sequences (the target site) immediately upstream (Gossen, 1992). tTAV accumulates to fatal levels at the larval stage (Gong et al., 2005), ostensibly due to sequestration of the transcriptional machinery (Morrison et al., 2009). Tetracycline inhibits this feedback loop, when supplied in larval diet. This disables the male-selection system and allows the strain to be propagated for mass-rearing (Fu et al., 2007).

The OX3864 sexing system can be applied as a population control system in its own right (Leftwich et al., 2014). All female progeny of released OX3864 homozygous males would die (half of all progeny); only heterozygous F_1 male offspring would survive. However, an issue with this strategy is that the F_2 progeny of heterozygous F_1 males and wild females would be 75% viable. This is because 25% of these F_2 progeny are heterozygous females, which die; all other progeny are viable. This incomplete sterility means that transgenes would propagate vertically in the field, through the viable male progeny of crosses between wild females and transgenic males. Furthermore, the OX3864 sexing system kills females as larvae, and allows all males to survive. Therefore, progeny resulting from the mating of a wild female to a released male, would still damage crops substantially. For these reasons, an engineered system for complete sterility (protamine-FokI expression) is superior, assuming it can mediate highly penetrant sterility and retain a fitness profile similar to wild males.

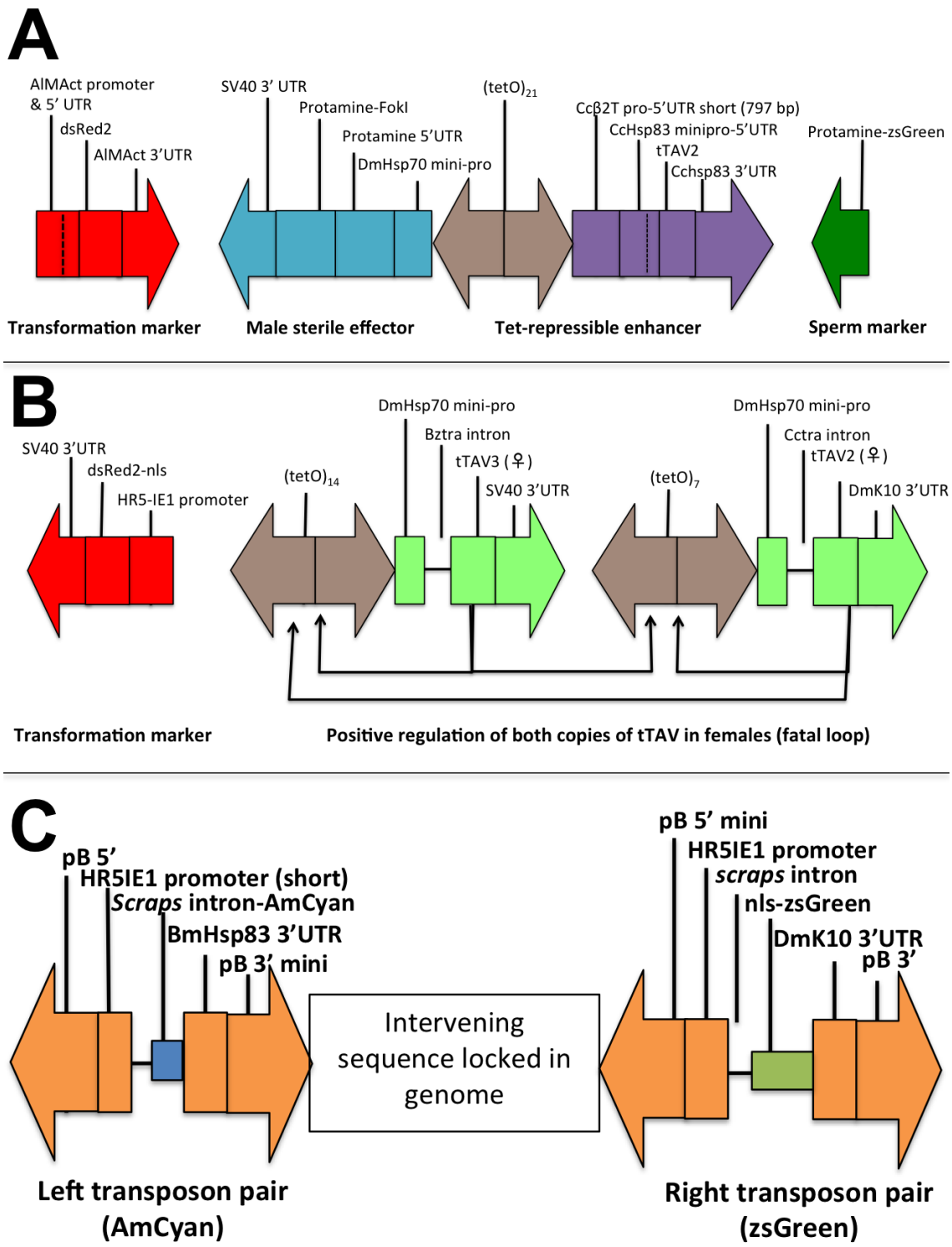


Figure 1.9. Engineered systems for repressible male sterility and genetic sexing (female removal). (A) First component: a transformation marker, repressible male sterility system expressing protamine-FokI in the male germline, and a fluorescent marking system for sperm nuclei (developed in Chapter 4). (**B**) Second component: tetracycline-repressible sexing strain that kills females. Two copies of tTAV are expressed specifically in females. A fatal positive feedback loop is generated by overexpression of tTAV. A double homozygous line would be generated by crossing both lines (A & B) together, to merge repressible male sterility and female removal. Note that both systems lack transposable ends in the final product. (**C**) Structure of the four ended piggyBac transposon system, which allows for the removal of transposable ends, after crossing to a transgenic line expressing transposase.

1.4.4 Fluorescent marking in the body and sperm

An effective population control strategy requires monitoring in the field, to determine the ratio of wild to released males (Vreysen, 2005). Released males can be marked with fluorescent dye (Hagler and Jackson, 2001). However, these marked males may be misscored if the dye dissipates (Robinson et al., 2004). This has been addressed by the constitutive expression of the fluorescent marker dsRed2 in muscle, using components of the Mexfly muscle actin gene, namely the promoter, 5'UTR and 3'UTR (Koukidou et al., 2016). This facilitates highly accurate scoring of transgenic individuals, in some instances by the naked eye. These transgenic strains could be further improved by engineering fluorescent marker expression in sperm nuclei, to monitor mating competitiveness in the field (Scolari et al., 2014). The frequency at which females mate released males can be scored by transfer of adhesive chemicals to females; the presence of irradiated sperm in the spermathecae (if morphologically different); or by PCR for sequences specific to the mass-reared strain (McInnis, 1993), (San Andres et al., 2007), (Armsworth et al., 2008). However, the scoring of fluorescently marked sperm would be expected to improve upon these methods.

A fluorescent sperm marking system would remove the necessity for an adhesive chemical and the risk of its dissipation in the field. It would be substantially easier to score fluorescent sperm than morphological variations induced in sperm, which are not present in all species, and which would be variable between individuals (Helinski and Knols, 2009). Finally, it would preclude the use of PCR screens and allow for a quantitative estimate of mating, possibly with greater accuracy than PCR. A series of fluorescent sperm markers linked to the male sterility effector (tetO-protamine-**mCherry**-FokI) or tetracycline-repressible transcriptional activator (tetO-Ccβ2tubulin promoter-Ccβ2tubulin 5'UTR-**dsRed2**-SV40 3'UTR) had been previously tested, but these did not reliably mark the nuclei of all sperm (Jin, 2011), (Asadi, 2013). The molecular components of these systems and their performance are fully detailed in the next chapter, where the development of a novel fluorescent sperm marking system is considered.

1.4.5 *piggyBac* expression constructs can be genomically immobilised

The components of this system have been engineered as *piggyBac* expression constructs integrated into the insect genome by transposition (Li et al., 2005). The expression profile of these features could be altered by re-transposition to a novel

insertion site (Wimmer, 2003). For instance, mobilisation of the transposon to transcriptionally silent heterochromatin could prevent the expression of the sterilant or the fluorescent markers (Markstein et al., 2008), potentially allowing the vertical transmission of transgenic sequences into the wild population. An additional concern is horizontal gene transfer, which could potentially introduce the transposon to other wild insect species (Lohe et al., 1995), (Silva et al., 2004). Therefore, the ability to fully immobilise the transgenic insertion would potentially enhance biosafety.

To achieve this end, Oxitec has developed a *piggyBac* vector system capable of complete excision of the transposable ends. This system applies a composite transposon with two pairs of *piggyBac* ends. A central element flanked by these two pairs contains the transgenic effectors required for the male sterility expression system. When crossed to a transgenic line expressing *piggyBac* transposase in the germline, the ends containing the transposable elements are mobilised away from the central element. This stabilises the transgenic effectors within the genome, because they are no longer associated with any transposon sequences (Dafa'alla et al., 2006). Excision of the ends can be screened for the absence of transposon sequences by PCR. Additionally, it is possible to design expression constructs with markers between each *piggyBac* end pair. This allows loss of ends to be screened visually (by the absence of fluorescent marker expression). Therefore, the application of the four-ended *piggyBac* system with a repressible protamine-FokI male sterility effector and fluorescent markers expressed in the body and sperm, could potentially provide an effective, novel and highly traceable method for the population control of Medfly, a pest which remains problematic despite decades of research.

Chapter 2 – Materials and methods

2.1 Molecular biology

2.1.1 Genomic DNA (gDNA) extraction

Two methods were applied. Generally, the PureLink Genomic DNA kit (Life Technologies K182000) was used with manufacturer's instructions. Alternatively, extraction was performed by high temperature, sodium hydroxide extraction in a thermocycler. Within a 96-well PCR plate, samples were placed in 100 mM NaOH (50 µl for adults and third instar larvae; 25 µl for a leg) and heated in a PCR thermocycler (99° C, 30 minutes). To neutralise, Solution B (50 mM HCl, 250 mM Tris-HCl [pH 8.5], 0.04% phenol red) was added: 2 µl for legs, 5 µl for larvae and adults. The second method was used where a large number of reactions needed to be performed quickly and in parallel.

2.1.2 RNA extraction

Total RNA of whole adult Medfly or the reproductive tract were extracted with the Total RNA kit (Norgen #17200) with manufacturer's instructions, for downstream use in RT-PCR, qRT-PCR, and 5' RACE.

2.1.3 Polymerase chain reaction (PCR)

All primers used in the study (**Table 8.1**) and the list of amplicons generated (**Tables 8.2-8.3**) are provided in the Appendix.

2.1.3.1 General PCR

The standard procedure was touchdown PCR with 35-40 cycles (10 cycles at 60° C annealing temperature, the remainder at 55° C). The annealing temperatures were adjusted where this did not provide adequate amplification. Q5 polymerase (NEB M0491) was used for cloning, 5'RACE and sequencing of CRISPR-induced mutations. BioTaq polymerase (PCR Biosystems BIO-21040) was used for all other applications. In both instances, PCR was performed by manufacturer's instructions. The general thermocycling programme is provided (**Table 2.1**).

Table 2.1 General PCR thermocycling programme

Step	Cycles	Description	Temp (° C)	Time (seconds)
1	1	Denaturation	95	120
2	10	Denaturation	95	15
3		Annealing	55-65	30
4		Extension	72	15 per kb
5	25-30	Denaturation	95	15
6		Annealing	55-65	30
7		Extension	72	15-90
8	1	Final extension	72	300
9	1	Holding	4	∞

2.1.3.2 Cloning and sequencing of PCR products

PCR products without non-specific bands were purified with the QiaQuick PCR purification kit (Qiagen #28104) by manufacturer's instructions. Where non-specific products were present, target bands were excised with a scalpel and purified with the QiaQuick gel extraction kit (Qiagen #28704). Where possible, PCR reactions were directly sequenced with an internal primer at Eurofins MWG Operon (Germany). Where this did not provide clean sequences, the products were blunt ended and cloned into the pJET vector with the CloneJET kit (Life Technologies K1231) and transformed into *E. coli* XL10 (Agilent Technologies) by heat shock-calcium chloride transformation, using manufacturer's instructions. Single colonies were screened by colony PCR in 10 µl reactions by standard methods, usually with pJET specific forward and reverse primers (TD1156 and TD1157). Cultures with the target insert were grown in in LB-ampicillin medium (100 µg/mL) with shaking incubation overnight (250 rpm, 37° C). Plasmids were mini-prepped with the GeneJET Plasmid Miniprep Kit (Life Technologies K0502) by manufacturer's instructions and sent for sequencing, as previously described.

2.1.3.3 Reverse transcription PCR (RT-PCR)

cDNA was synthesised from total RNA extractions with the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher K1621) by manufacturer's instructions. A 1:1 ratio of random hexamer primers and poly(A) annealing primers was applied. Thereafter, RT-PCR was performed in 20 µl reactions with 1 µl of cDNA template, generally under the standard conditions previously described for genotyping PCR (35-40 cycles: 10 at 60° C, the remainder at 55° C). For semi-quantitative RT-PCR, samples were aliquoted at 25, 30, 35 and 40 cycles (the first 10 cycles were performed at 60° C annealing temperature, and all subsequent cycles at 55° C annealing temperature).

Constitutively expressed control genes (internal standards) were amplified, Medfly alcohol dehydrogenase (*adh*) or ribosomal protein P0 (*Cc-RpP0*).

2.1.3.4 Quantitative RT-PCR (qRT-PCR)

The SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (ThermoFisher 12574030) was used with manufacturer's instructions. The thermocycling programme is provided (**Table 2.2**). Rox was used as a reference dye, and the C_t values were first normalised to Rox. Dissociation curves from the melt curve analysis were monitored to identify primer dimers or non-specific product. Relative gene expression was calculated with the $\Delta\Delta C_t$ method (Yuan et al., 2006). For each sample, C_t values for the transgene amplicon were normalised to those of the endogenous control amplicon *Cc-Rp17S* (by subtraction of the C_t for the transgene from the C_t for the control gene), to derive the ΔC_t. Thereafter, a reference experimental group was chosen as a calibrator (a transgenic strain with minimal or no expression). The average ΔC_t for each experimental group was normalised to the average ΔC_t of the reference group (by subtraction of the ΔC_t of each experimental group from the ΔC_t of the control group, to derive the $\Delta\Delta C_t$. To express fold-change, the measurements were log transformed: $2^{-\Delta\Delta Ct}$. To express uncertainty, standard deviation or standard error ("s") were calculated from the C_t values of each experimental group, and subsequently log transformed: $2^{-\Delta\Delta Ct \pm s}$.

Table 2.2 qRT-PCR thermocycling programme

Step	Cycles	Description	Temp (° C)	Time (seconds)
1	1	cDNA synthesis	50	180
2		Denaturation	95	300
3	40	Denaturation	95	15
4		Annealing	60	30
5		Extension	40	60
6	1	Melt curve analysis	95	60
7			55	30
8			95 (collections at every 0.5° C)	30

2.1.3.5 Inverse PCR and adapter ligation PCR

To isolate gDNA flanking transposon insertions, inverse PCR was performed with a modified protocol from Buckholz et al. Two sets of PCR reactions were performed at each end, to isolate gDNA upstream (5') and downstream (3') of the transgenic insertion site. Digestions were performed with SauAI, HpyCh4IV, TaqαI and

HaeIII (5’ end) or HhaI, AvaII, HaeIII and MspI (3’ end) to yield short fragments spanning the genomic DNA and transgenic expression construct. The restriction fragments were circularised by blunt-ending and ligation with the CloneJET kit (Life Technologies K1231), following manufacturer’s instructions. PCR reactions were performed in 20 µl volumes with BioTaq polymerase buffer, 0.5 µM primers, BSA (0.1 mg/ml), BioTaq polymerase, and template (first reaction: 5 µl of the ligated restriction fragments; second reaction: 0.5 µl of the first PCR). The thermocycling programme is provided (**Table 2.3**). The dominant band of the second (nested) reaction from each end (5’ and 3’) was gel excised, blunt ended, cloned into the pJET vector and transformed into *E. coli* XL-10; all as previously described (**Section 2.1.3.2**). Colonies were screened, grown overnight in LB-ampicillin culture, and plasmids were miniprepped and sequenced as previously described, to identify the flanking sequences upstream and downstream of the insertion.

Table 2.3 Inverse PCR thermocycling programme

Step	Cycles	Description	Temp (° C)	Time (seconds)
1	1	Denaturation	95	300
2	34	Denaturation	95	30
3		Annealing	55	60
4		Extension	72	60
5	1	Final extension	72	120
6	1	Holding	4	∞

Alternatively, it is possible to restriction digest gDNA and ligate an adapter fragment to the 5’ and 3’ end. Three nested PCRs (each with an adapter-specific and transposon-specific primer) are thereafter performed, prior to sequencing across the adapter, flanking gDNA, and transgenic expression construct. This was used by a colleague (Caroline Phillips, Oxitec) to isolate the OX4718A-resolved flanking sequence.

2.1.3.6 Rapid amplification of cDNA ends (RACE)

5’ RACE was performed to identify the transcriptional start site (TSS) of Medfly protamine-like genes. The SMARTer RACE 5'/3' Kit (Clontech 634858) was used with manufacturer’s instructions. cDNA is synthesised with poly(A) annealing primers and a proprietary adapter sequence is added to the 5’ terminus of the cDNA by the terminal transferase activity of the enzyme, labelling the putative TSS. The cDNA of the target gene was amplified by two nested PCRs with Q5 polymerase, each with a gene-specific reverse primer and the proprietary universal forward primer (UFP) mix. The standard

touchdown thermocycling conditions were applied. The dominant band of the first PCR reaction (pre-nesting) was processed as previously described: gel purified, cloned into the pJET vector, transformed into *E. coli* XL-10; thereafter, colonies were screened for the presence of the insert by colony PCR with gene-specific primers. Colonies with the correct insert were grown overnight in LB-ampicillin medium, and the plasmids miniprepped. Multiple clones were sequenced with a gene-specific reverse primer. Sequences were aligned and trimmed to the terminus of the proprietary adapter, to identify the putative TSS. Thereafter, the results were compared to those obtained via high throughput transcript sequencing (NCBI, 2016).

2.1.3.7 Synthesis of CRISPR sgRNAs

All CRISPR experiments utilised the Cas9 nuclease from *S. pyogenes* (Sp-Cas9). In theory, any site can be targeted as long as an nGG protospacer adjacent motif (PAM) follows the target sequence (Brouns et al., 2008). To clarify, the target genomic sequence is sense, relative to the sgRNA. It is possible to target other motifs, but the efficiency of cutting is typically reduced (Zhang et al., 2014). Target-specific CRISPR sgRNAs were synthesised by no-template PCR, *in vitro* transcription, and phenol-chloroform extraction, essentially as previously reported (Bassett et al., 2013). The no-template PCR applies a forward primer specific to the target and a common reverse primer (SS1713); the product is a primer dimer. The degenerate forward primer: GAAAT**TAATACGACTCACTATA***[N]*$_{20}$GTTTTAGAGCTAGAAATAGC comprises the T7 promoter (bold), a 20 nucleotide seed sequence that is identical or nearly identical to the genomic target (italics), and a complementarity region for the reverse primer (underlined). To facilitate efficient in vitro transcription from the T7 promoter, the two 5' nucleotides of the seed sequence were changed to GG (Kuzmine et al., 2003). In most cases, this did not match the genomic target. However, mismatches at the 5' end are well-tolerated in CRISPR platforms (Cradick et al., 2013); these slightly off-target sgRNAs were shown to mediate efficient mutagenesis (**Chapter 6**). Reactions were comprised of: Q5 buffer, target specific forward primer (0.4 μM), common reverse primer SS1713 (0.4 μM), dNTPs (0.2 mM) and Q5 polymerase. Reactions were amplified for 35 cycles with the standard touchdown PCR method described previously (**Table 2.1**).

The product was directly purified with the Fermentas GeneJet PCR Purification Kit (ThermoScientific K0701) and 500 ng of this DNA intermediate was converted to

sgRNA by *in vitro* transcription from the T7 promoter, with the Ambion Megashortscript T7 kit (ThermoScientific AM1354). The sgRNAs were purified by phenol-chloroform extraction. To each reaction, 150 µl phenol-chloroform was added prior to centrifugation (14600 rpm, 5 min). The aqueous phase was transferred to 150 µl chloroform and centrifuged (14600 rpm, 5 min). The aqueous phase was transferred to 300 µl ethanol and incubated (15 min, -20° C) prior to centrifugation (14000 rpm, 15 min, 4° C). The supernatant was removed and the pellet was resuspended to 1 µg/µl RNA, and stored at -80° C. A list of sgRNA sequences is provided in the Appendix (**Table 8.4**).

To pre-validate the cutting activity of sgRNAs, an assay was performed with manufacturer's instructions (PNA Biotech). Reactions (10 µl) were comprised of 500 ng Cas9 protein, 250 ng sgRNA, 200 ng of target PCR product or linearised plasmid, NEB Buffer 3 and BSA (0.1 mg/ml). The reaction was incubated at 37° C for 30-60 minutes and the reaction stopped with 4 µg RNaseA (10 min, 37° C) prior to addition of 1 µl stop solution (30% glycerol, 1.2% SDS, 250 mM EDTA pH 8) and incubation (10 min, 37° C). Cleavage of the target fragment was visualised by gel electrophoresis.

2.1.4 Computational and statistical analysis

DNA and protein sequences were aligned for sequence homology with T-Coffee or M-Coffee Multiple Sequence Alignment (MSA). Protein sequences were assessed with EMBL Interpro. The TSS of genes of interest were identified from high-throughput transcript sequencing data (FlyBase or NCBI) or 5'RACE. All statistical analyses were performed with the standard R package (version 3.3.0, Mac OS X). Sequence alignment and expression construct design were performed with SnapGene software (GSL Biotech, v2.6.2, Mac OS X). To compare homologous proteins, a similarity index was calculated, as the ratio of conserved residues (identical or conservative substitutions) to the mean number of residues:

$$\frac{A}{(B+C)/2} * 100$$

A: Identical residues or conservative substitutions

B: Residues in first sequence

C: Residues in second sequence

2.2 Medfly rearing

Rearing was generally as previously described (Saul, 1982a). Typically, conditions were 26° ± 2° C and 50% ± 10% relative humidity with a photoperiod of 12 hours light (07:00-19:00) and 12 hours dark (19:00-07:00). Rearing was occasionally performed at 18° ± 1° C, 20° ± 1° C or 30° ± 1° C to alter the rate of development, which is increased by higher temperatures and decreased by lower temperatures (Duyck and Quilici, 2002). The WT culture was the Toliman strain from Guatemala (Lance et al., 2000). It has been in continuous culture at Oxitec since 2004 (about 175 generations). All transgenic lines were derived by *piggyBac* transformation of this background.

Adults were cultured in plastic cages with a mesh-coated surface for oviposition and separate vessels for water and adult diet (80% sucrose [Tate & Lyle] and 20% autolysed yeast [Fisher 10255153]). Flies requiring tetracycline to repress transcription of transgenic elements regulated by tetO-minimal promoter (Gossen, 1992) were supplied water with 100 µg/ml tetracycline (Sigma-Aldrich T7660). Five types of cages were used for culturing. Four were developed at Oxitec (small, medium, large and extra large) and one was commercially obtained (BugDorm cages, Megaview Science). Images of the cages (**Figure 2.1**) and their properties (**Table 2.4**) are provided. WT flies and homozygous lines were reared at a 1:1 sex ratio, in extra-large cages and medium/large cages, respectively. Non-homozygous transgenic lines were usually maintained by backcrossing transgenic males to WT females at a 1:2 ratio (♂:♀), in small cages. Mating was allowed for at least four days. BugDorm cages were used for competition assays. Small cages were used for all other experiments (unless noted otherwise).

Table 2.4: Properties of cages for adult rearing

Cage	Dimensions (cm, length x width x height)	Adult population
Small	10 x 10 x 10	1-40
Medium	15 x 13 x13	30-150
Large	18 x 17 x 17	100-300
Extra large	24 x 50 x 26	500-1500

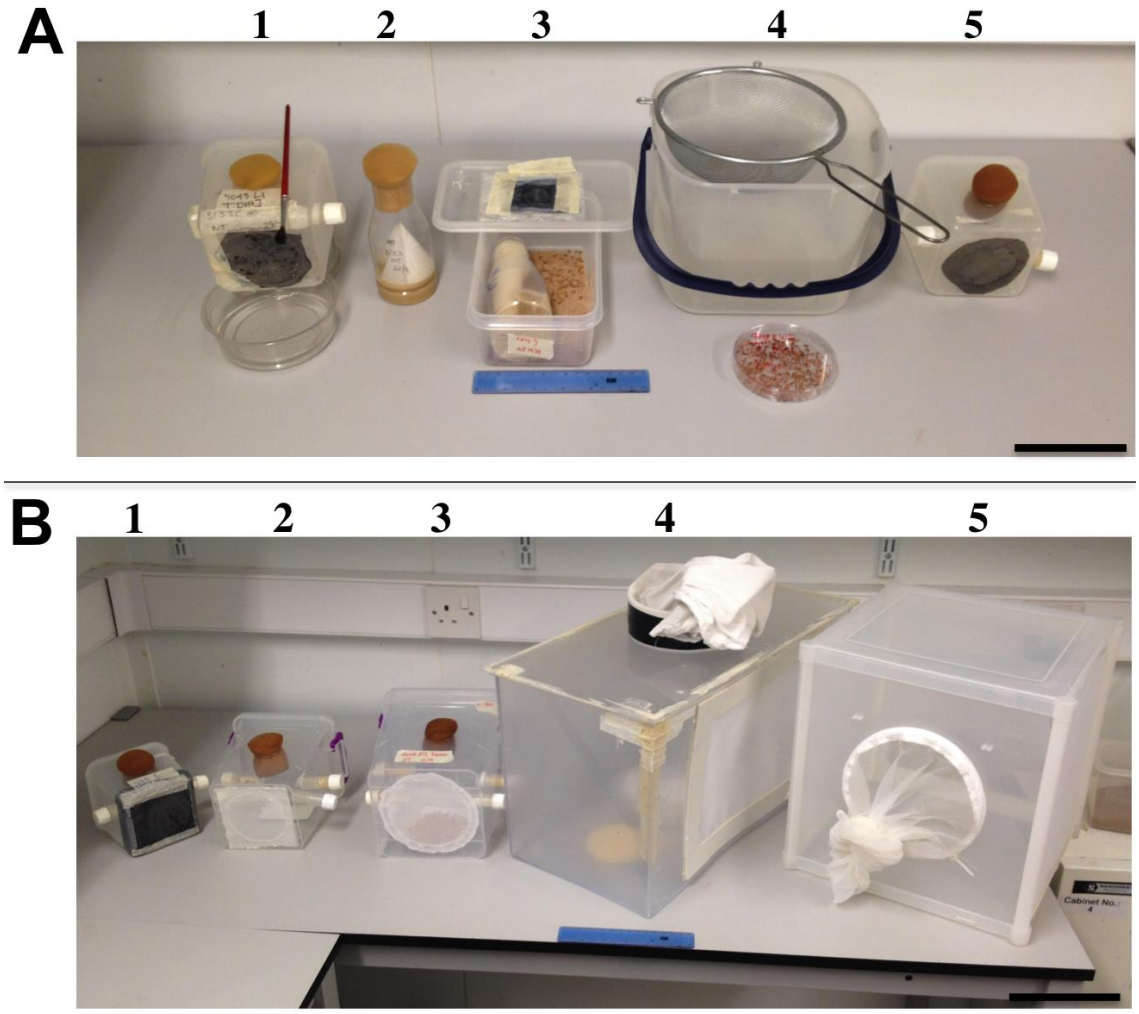


Figure 2.1. Laboratory rearing of Medfly. (**A**) Culturing at each life stage. Eggs fall into a vessel with water (1). A QL125 filter paper with eggs is added to a bottle with larval diet (2). A cone of QL135 filter paper is added to bottles with third instar larvae, then enclosed in a box with sand (3). Pupae are separated from sand with a coarse sieve and screened for fluorescent marker expression (4). Adults are crossed to repeat the cycle (5). (**B**) Cages for Medfly rearing in small (1), medium (2), large (3), and extra large (4) sizes; and the commercially obtained BugDorm cage (5). In the small, medium and large cages food is provided in half-cylindrical feeders; water is provided in cylindrical tubes stuffed with cotton. In the extra large and Bugdorm cages, water is supplied in bowls with a sponge and food in Petri dishes. Mesh-coated surfaces allow oviposition, in all cages except Bugdorms. Scale bars: 10 cm.

Females of the Oxitec Toliman strain accept mates from the second or third day post-eclosion, and begin to lay eggs on the third day. Eggs are brushed from the oviposition mesh into a collection tray filled with distilled water. Eggs were collected between days 4-10, filtered through Fisherbrand QL125 paper (Fisherbrand 11754233) and folded to fit in 250 ml plastic bottles (F L Plastics, UK) with larval diet (66.5 g maize meal [Holland & Barrett "Amazing Maize Meal"], 73 g sucrose [Tate & Lyle], 46.5 g autolysed yeast [Fisher 10255153], 10 g agar [BTP Drewitt], 2.5 g methyl-4-hydroxybenzoate [Sigma-Aldrich W271004], with dH_2O to 1 L). The sucrose content was 7.3% and the protein content 3%, calculated from the manufacturer's specification

of the maize meal and an average 56% protein content of yeast powder (Bayarjargal et al., 2014). Tetracycline was added at 100 μg/ml, where required to suppress tetO-minimal promoter regulated transgenic elements (Gossen, 1992).

Five days after seeding, bottles were enclosed within sealed plastic boxes (18 cm x 11 cm x 7 cm), with a mesh-covered airhole. Sand (Argos 365/0574) sterilised by dry heating in an oven (121° C, 4 hours), was added to 1 cm depth as a substrate for pupation (Vargas et al., 1986). A cone was formed from five sheets of QL135 filter paper (Fisher 11704213) and pressed to the bottom of the bottle, to absorb excess moisture and aid larval exit. Pupae were collected through a coarse sieve 7-10 days later. Transgenic lines were screened by microscopy for the presence of the fluorescent transformation marker (Olympus SZX12 microscope and Olympus U-RFL-T fluorescent burner**)**. Adult flies were anaesthetised on ice and collected no later than 24 hours (26° C) or 48 hours (20° C) post-eclosion, to ensure virginity.

2.3 Microinjection and transgenesis

2.3.1 Expression constructs used in the study

A list of expression constructs evaluated in the study and the phenotypes observed after transgenesis, is provided in the Appendix (**Table 8.5**). Diagrams of the constructs are provided in **Figure 2.2**; these are repeated in the results section where phenotypic assessment was performed. A loose copy of this figure is provided, for reference throughout the thesis.

2.3.1.1 *piggyBac* vectors

All genomically integrated expression constructs were *piggyBac* vectors. Generally, standard two-ended vectors were applied (Handler et al., 1998). For product candidates, four-ended vectors that allow removal of the sequences required for transposition were used. Remobilisation of the two pairs of *piggyBac* ends effectively locks the construct at the genomic insertion site (Dafa'alla et al., 2006). OX5140, OX5150, OX5182, OX5184, OX5186, and OX5173 were synthesised by the author by standard methods (PCR, restriction digest and ligation; or Gibson Assembly). All other constructs were kindly synthesised by the Oxitec Molecular Team (Caroline Phillips, Tarig Dafa'alla, Sarah Scaife, Andrea Miles & Tabi Jenkins) or former students (Li Jin, Michal Bilski & Romisa Asadi). Lines of OX3133, OX3671, OX4014, OX4282, OX4353, OX4705, OX4718 and OX4801 were assessed prior to the initiation of this studentship. All other lines were obtained and assessed by the author. Assistance from colleagues was generally provided for microinjection, usually one or two persons (Romisa Asadi, Thea Marubbi, Dylan Noone, Christa Kistenpfennig, Charilaos Megas or Carlos Pedraz) to line up eggs in an appropriate orientation for microinjection. This was the case for microinjections of all piggyBac constructs and CRISPR sgRNAs in this study, with the exception of OX5182, OX5184, OX5186, OX5134, OX5154 and OX5173.

2.3.1.2 CRISPR expression constructs and materials

Recombinant Cas9 protein from *S. pyogenes* was purchased from PNA Biotech (CP01). OX5173 transgenic lines, which express nanos-regulated Cas9 in the germline, were obtained by *piggyBac* transgenesis. Target-specific CRISPR sgRNAs were synthesised as previously described (**Section 2.1.3.7**); the genomic targets of all sgRNAs are provided in the Appendix (**Table 8.4**) and referenced in the relevant Results section (**Chapter 6**).

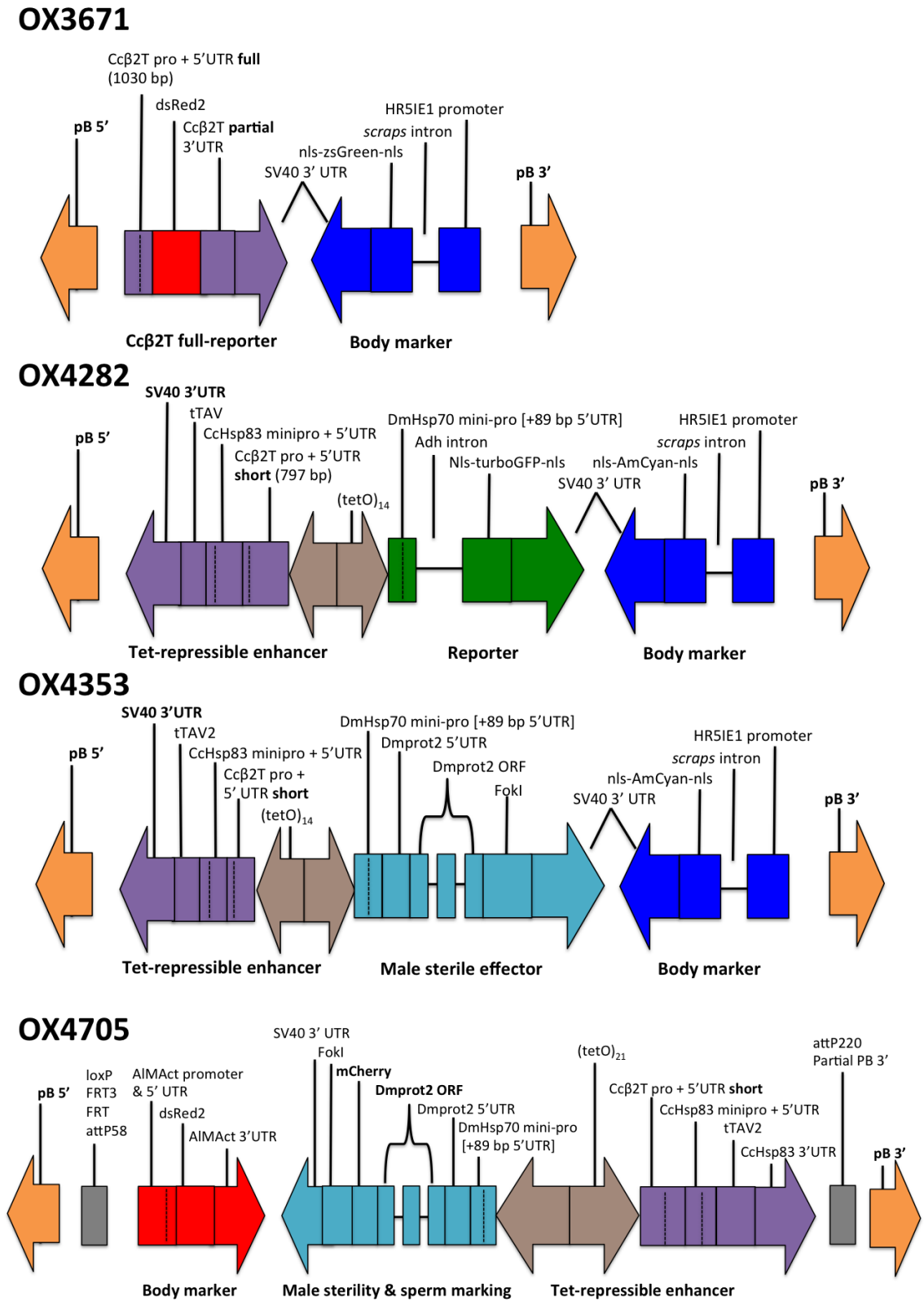


Figure 2.2 (Part 1). Expression constructs used in the study. All diagrams are repeated in the relevant results section with a full description of their contents. OX3671 & OX4282 were reporter constructs to test Ccβ2tubulin-derived expression systems. Pre-meiotic transcription and translation of tTAV in the male germline was required to activate sufficient pre-meiotic transcription of a protamine-FokI male sterility effector. The Ccβ2tubulin-Cchsp83-tTAV element from OX4282, was used in all subsequent constructs. OX4353 & OX4705 were constructs for tetracycline-repressible male sterility. The OX4705 male sterility effector has a fused fluorescent sperm marking system (without SG4 linkers). Dashes indicate promoter-5'UTR boundary.

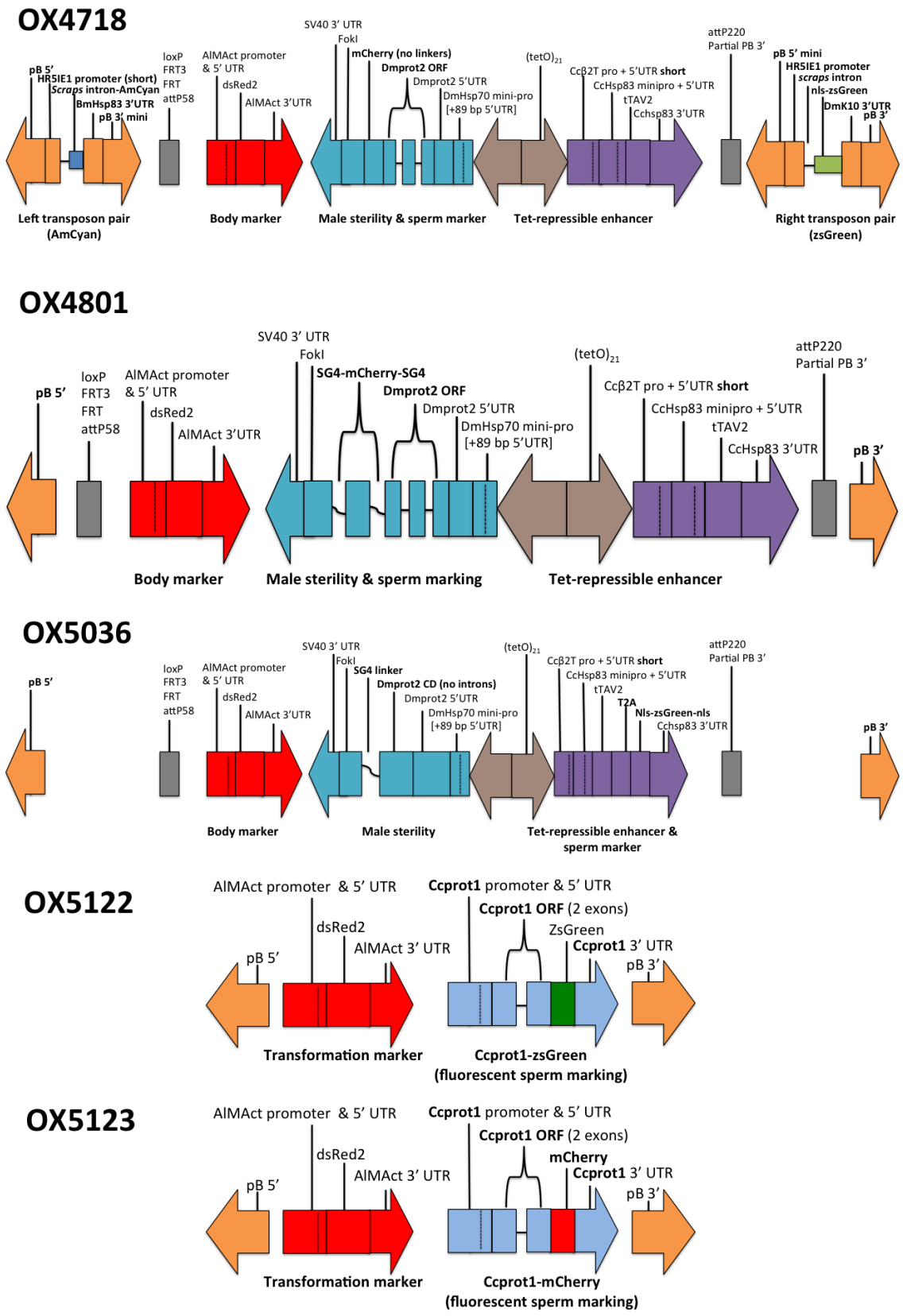


Figure 2.2 (Part 2). Expression constructs used in the study. OX4718, OX4801 & OX5036 were constructs for tetracycline-repressible male sterility with fluorescent sperm marking systems. The fluorescent sperm marker was fused to the sterility effector in OX4801 (with SG4 linkers) and OX4718 (without SG4 linkers). In OX5036, tTAV and nls-zsGreen-nls (the fluorescent sperm marking system) were translated from the same mRNA as two polypeptides, separated at the T2A sequence. OX5122 & OX5123 were constructs with protamine-fluorescent marker fusions (Ccprot1-zsGreen or Ccprot1-mCherry) for fluorescent sperm marking. Dashes indicate promoter-5'UTR boundary.

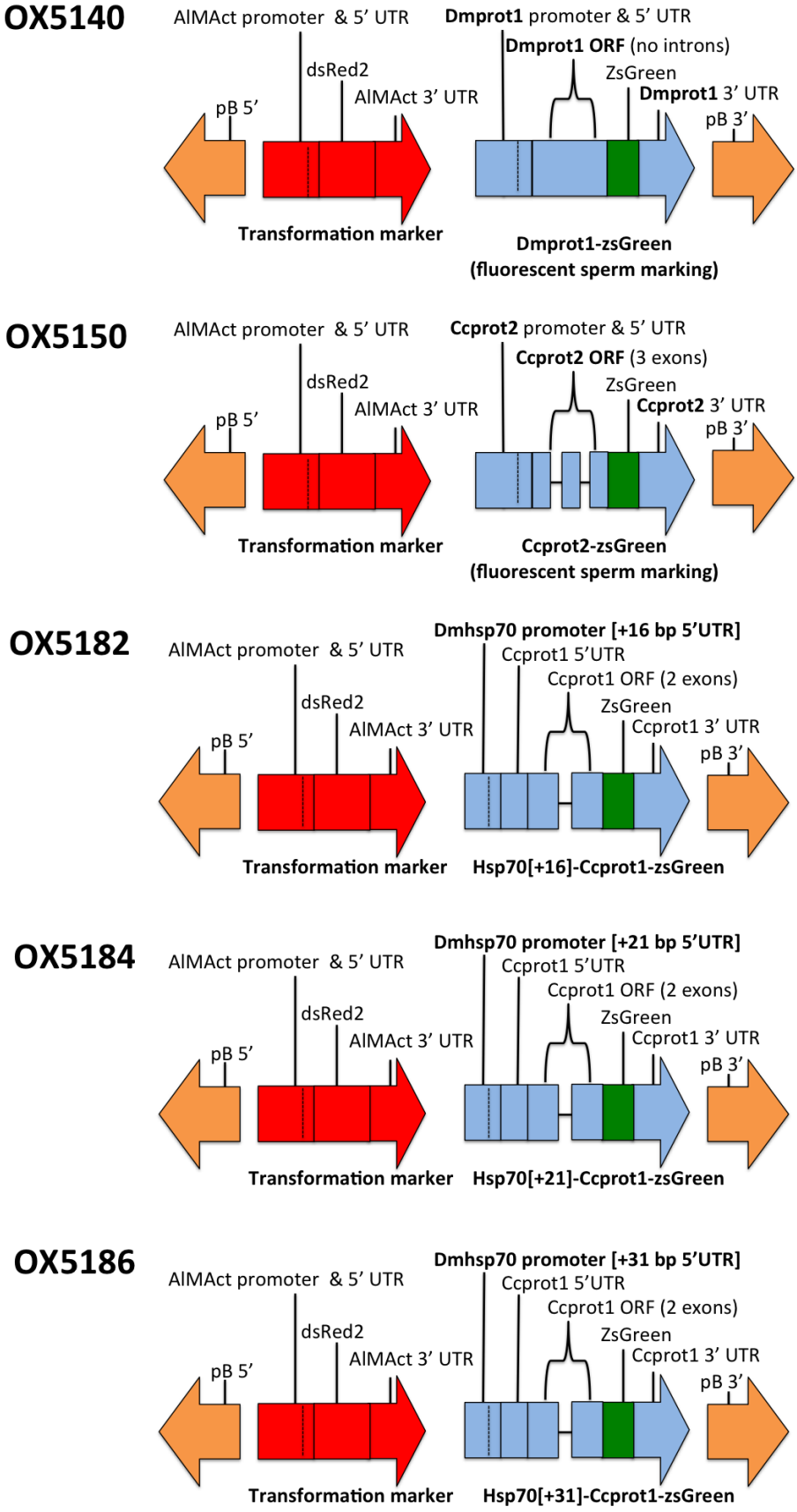


Figure 2.2 (Part 3). Expression constructs used in the study. OX5140 & OX5150 were constructs with protamine-fluorescent marker fusions for fluorescent sperm marking (Dmprot1-zsGreen or Ccprot2-zsGreen). OX5182, OX5184 & OX5186 were DmHsp70 promoter-Ccprot1-zsGreen reporter fusions to verify that the Ccprot1 5'UTR would mediate translational repression when applied to regulate effectors for repressible male sterility. They varied in the length of Dmhsp70 5'UTR included in the fusion to the Ccprot1 5'UTR. Dashes indicate promoter-5'UTR boundary.

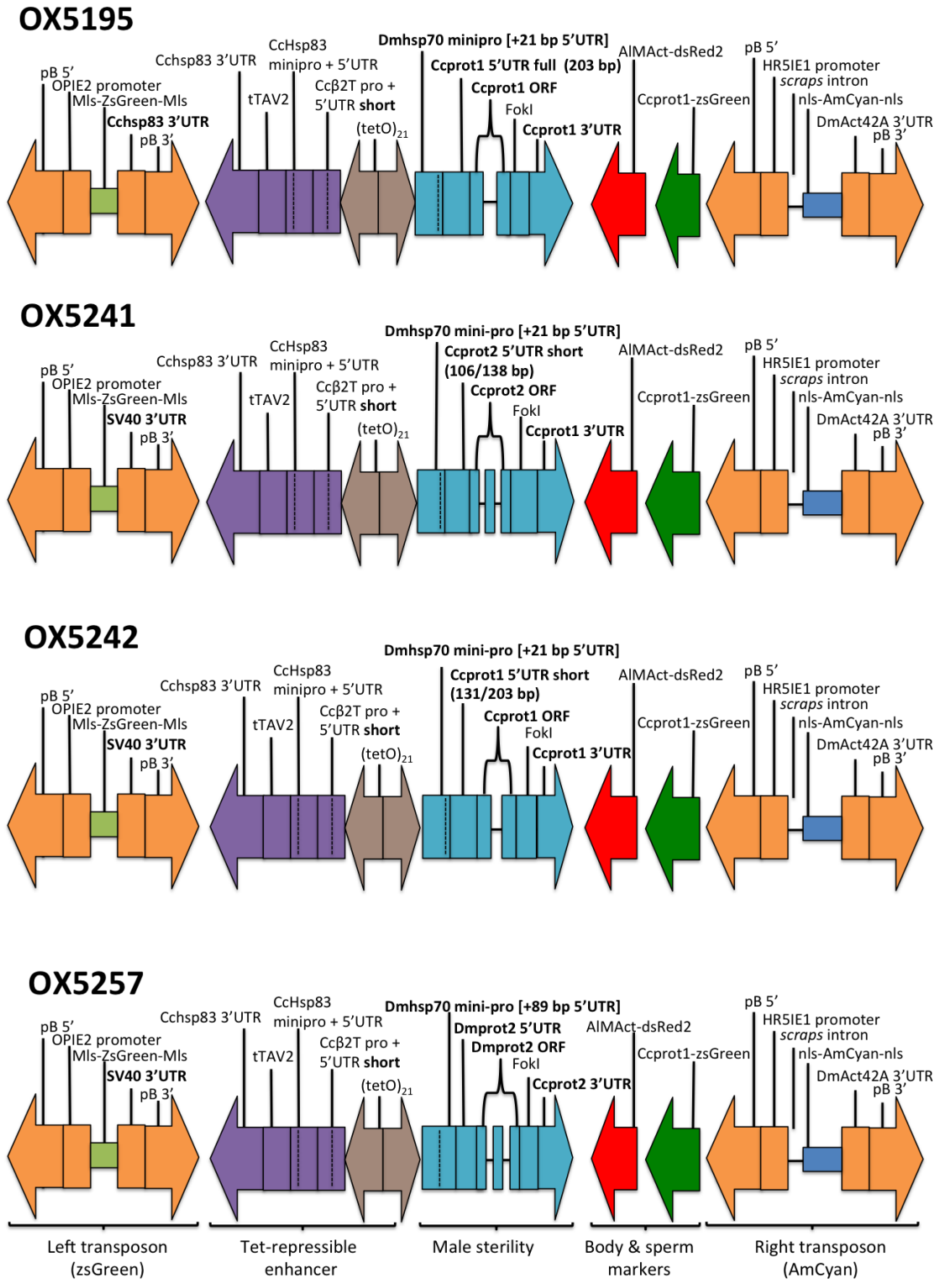


Figure 2.2 (Part 4). Expression constructs used in the study. OX5195, OX5241, OX5242 & OX5257 were constructs for tetracycline-repressible male sterility with fluorescent sperm marking systems. Four systems for repressible male sterility were tested: OX5195 (Ccprot1-full 5' UTR-FokI); OX5241 (Ccprot2-short 5'UTR-FokI); OX5242 (Ccprot1-short 5'UTR-FokI); OX5257 (Dmprot2-chimeric-new-FokI). All constructs incorporated a fluorescent marker translated in the body (AlMAct promoter-AlMAct 5' UTR-dsRed2-AlMAct 3' UTR) and a second fluorescent marker translated in sperm nuclei (Ccprot1 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1 3'UTR). Dashes indicate promoter-5'UTR boundary.

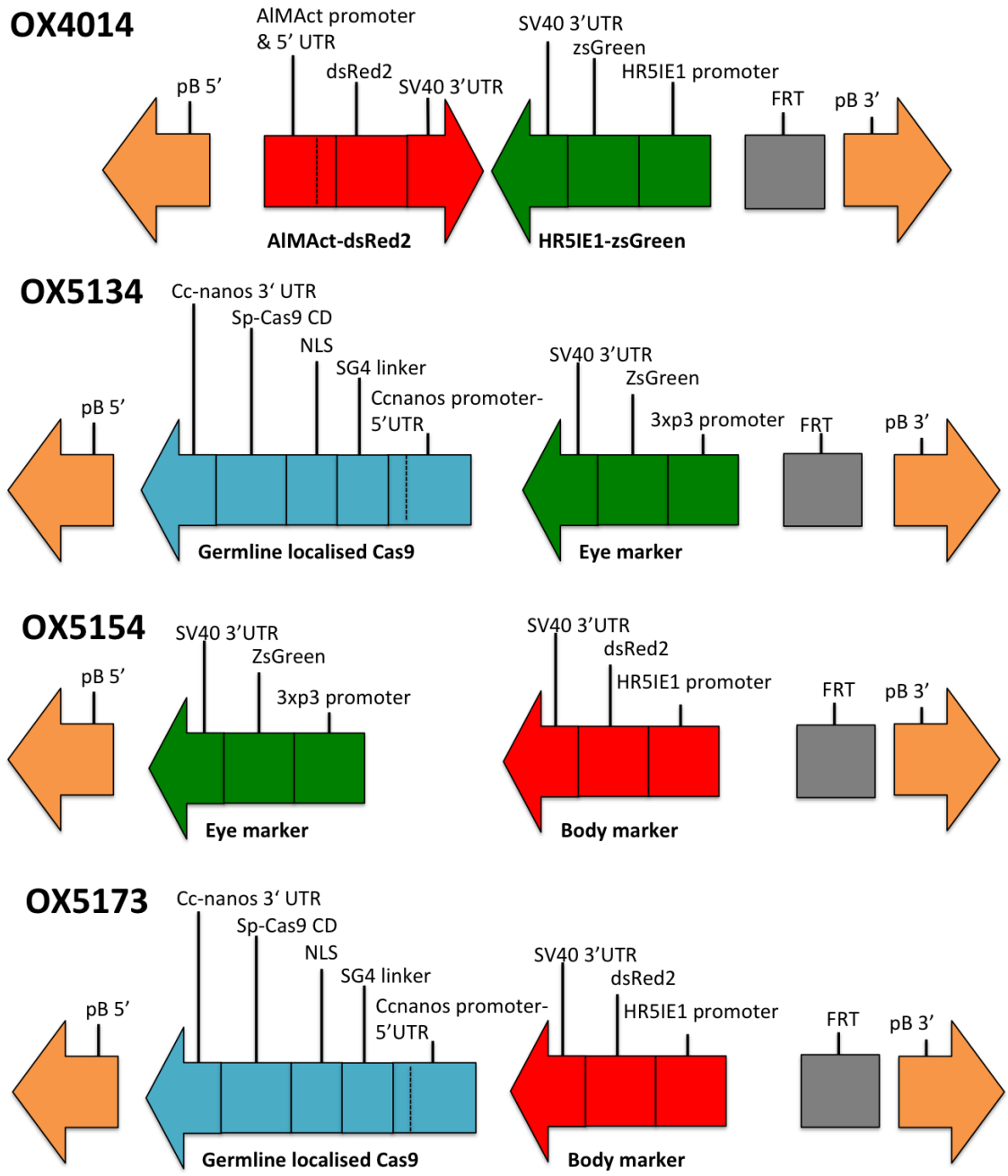


Figure 2.2 (Part 5). Expression constructs used in the study. OX4014 is a piggyBac vector with multiple fluorescent markers, used in CRISPR mutagenesis experiments. OX5134 and OX5173 were designed to express Cas9 in the germline (nanos-Cas9). Lines of OX5134 were not obtained. OX5154 was a double transformation marker construct to confirm that 3xP3-zsGreen (also used in OX5134) was not functional, therefore explaining why OX5134 transgenic lines were not obtained. Dashes indicate promoter-5'UTR boundary.

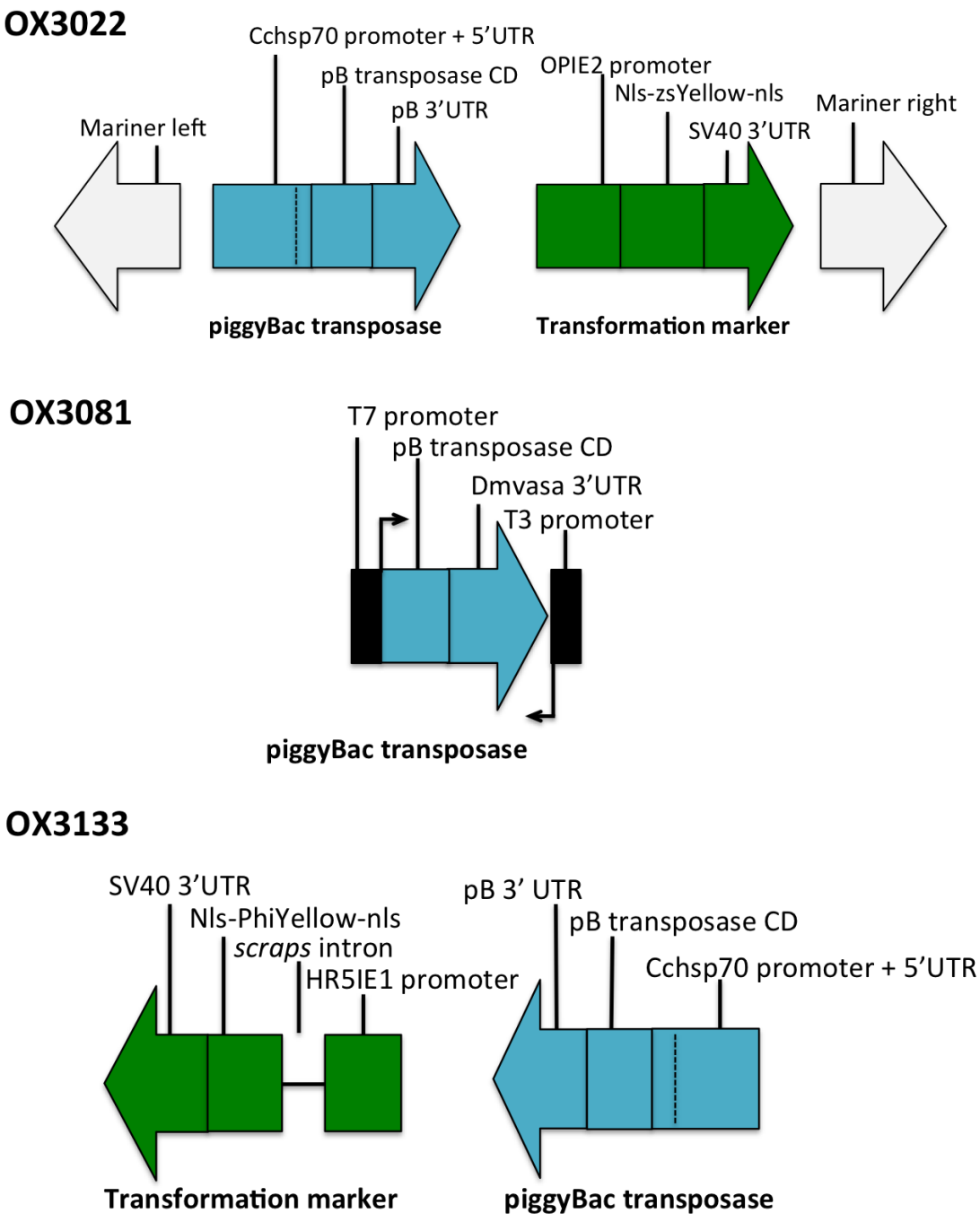


Figure 2.2 (Part 6). Expression constructs used in the study. OX3022 and OX3081 were helper constructs for piggyBac transgenesis, encoding pB transposase. OX3022 was directly injected; OX3081 was converted to mRNA by in vitro transcription from the T7 promoter. OX3022 contains transposable ends (mariner) and a transformation marker, but transgenic lines were not made for this construct. Transgenic lines of OX3133 were used to mobilise ("resolve") the piggyBac end pairs in four-ended constructs, thereby locking the central element encoding the transgenic effectors in the genome. These OX3133 lines also lack pB ends (it was a 4-ended vector, resolved by the transposase encoded on the construct). Dashes indicate promoter-5'UTR boundary.

2.3.2 Microinjection of embryos

For *piggyBac* transgenesis, buffered injection mixes (0.1 mM sodium phosphate pH 7.8, 5 mM potassium chloride) were prepared with the helper plasmid (OX3022) and the *piggyBac* vector at 300 ng/µl and 600 ng/µl, respectively. Additional transposase was occasionally supplied as mRNA (OX3081) at 300 ng/µl. The components of all CRISPR injection mixes are outlined in the relevant results section. Mixes were centrifuged (15 min, 14600 rpm), and the upper (soluble) fraction removed and assessed by gel electrophoresis.

WT embryos were injected for *piggyBac* transgenesis. For CRISPR studies, the injection genotype was the fluorescent marker expressing line OX4014A-homozygous (dsRed2 and zsGreen); all studies involved the mutagenesis of fluorescent marker or *piggyBac* sequences. Four to ten days after eclosion, embryos were collected in cycles of 30-90 minutes, to avoid injection of post-blastodermal eggs (Beech et al., 2006). Embryos were passed through a fine sieve to remove water, and treated with a ten-fold dilution of sodium hypochlorite solution (Sigma-Aldrich 425044) until visibly dechorionated (about 90 seconds). With a fine needle, embryos were transferred to cover slips with glue (Scotch double-sided tape dissolved in heptane).

To prevent bursting when injected, embryos were desiccated at 37 °C until they appeared suitably dry (3-7 minutes) and coated with a 7:1 mixture of halocarbon oils 700 and 27 (Sigma-Aldrich H8898 and H8773), prior to microinjection at the posterior pole. Femtotips II needles (Eppendorf) were used with the following microinjector setup: Eclipse Ti-S microscope (Nikon), Transferman NK2 micromanipulator (Eppendorf), FemtoJet pressure supply (Eppendorf), and air compressor (Jun-Air 1109020). The operating pressure of the microinjector (usually 30-200 hPa) was adjusted to suit the needle width and mix viscosity. The coverslips with injected embryos were placed in Petri dishes with agar-fruit juice (15 g agar, 12.5 g sucrose, 2 g methyl-4-hydroxybenzoate, 250 ml fruit juice, 750 ml H_2O) and a moist Whatman grade 3 filter paper in the lid, prior to sealing with Parafilm.

With a fine needle or paintbrush, surviving larvae were transferred to a bottle with standard larval diet or a Petri dish with enriched medium (158 g corn grits, 84 g sucrose, 64 g autolysed yeast powder, 1 g sodium benzoate, 2.3 g methyl-4-hydroxybenzoate, 1 g guar gum, 6 g citric acid, 84 g wheat germ, 1 g vitamin mix

#6265 [BioServ], 65 g cellulose, 550 ml water, 0.6 ml 37% hydrochloric acid). After larvae developed to the third instar, the vessel with diet was transferred to a box with sand to allow pupation. For *piggyBac* transgenesis, pupae that survived microinjection were screened for transient expression of the fluorescent marker (HR5IE1-dsRed2, 3xp3-zsGreen or AlMAct-dsRed2), to confirm successful microinjection.

2.3.3 Establishment of transgenic lines and assessment of the transgenic copy number and insertion site

To isolate transgenic lines, microinjection survivors (G_o adults) were backcrossed to WT in pools comprised of G_o adults of a single sex. The number of individuals crossed in each pool was adjusted to reflect the number of G_o adults obtained and the number of transgenic lines required (all crosses are stated in the relevant results section). Usually, 5-15 G_o males were crossed to 15-30 WT females. For the reciprocal crosses, 5-30 G_o females were crossed to 10-20 WT males. G_1 progeny with transgenic insertion(s) were identified by expression of the fluorescent marker under a stereoscope (Olympus SZX12 microscope and U-RFL-T fluorescent burner). Single transgenic G_1 individuals of each pool were backcrossed to WT (2 males or 3 females). For CRISPR experiments, G_0 injection survivors were backcrossed to WT as previously described and G_1 progeny were screened by fluorescence microscopy for phenotypic reversion (loss of the fluorescent markers).

To identify the copy number and insertion site of *piggyBac* expression constructs (autosomal or sex-linked), G_2 or G_3 progeny were assessed by Mendelian analysis of the inheritance of the dominant marker. Medfly have five autosomes and a pair of XX or XY sex chromosomes (Zacharopoulou, 1990). Therefore, for a single insertion, or multiple insertions to the same chromosome, the proportion of transgenic progeny is 0.5. If the vector inserts into both homologous chromosomes, all progeny are marked at the G_2 stage, but not in subsequent generations. For multiple insertions to heterologous chromosomes, each additional insertion increases the proportion of marker-expressing progeny by 50% of the previous value. This is summarised by: $1-(1/2^n)$, where n is the number of heterologous chromosomes with transgenic insertions (**Table 2.5**). To assess sex-linkage, the sex ratio of transgenic individuals is scored. The outcome is dependent on the sex of the transgenic parent. For a male individual with a single insertion, transgenic progeny are male-only if Y-linked, female-only if X-linked, and bisexual if autosomal. For a female individual with a single insertion, the transgenic

progeny are bisexual regardless of the insertion site. However, if transgenic males of the subsequent generation are crossed, an X-linked insertion is reflected by the absence of male transgenic progeny (**Figure 2.3**).

Table 2.5 Proportion of marker-expressing progeny for multiple insertions to heterologous chromosomes

Heterologous chromosomes with insertions	Proportion of marker-expressing progeny
1	0.5
2	0.75
3	0.88
4	0.94
5	0.97
6	0.98

Single and double insertions were obtained, but an individual with three or more insertions was never observed in this study.

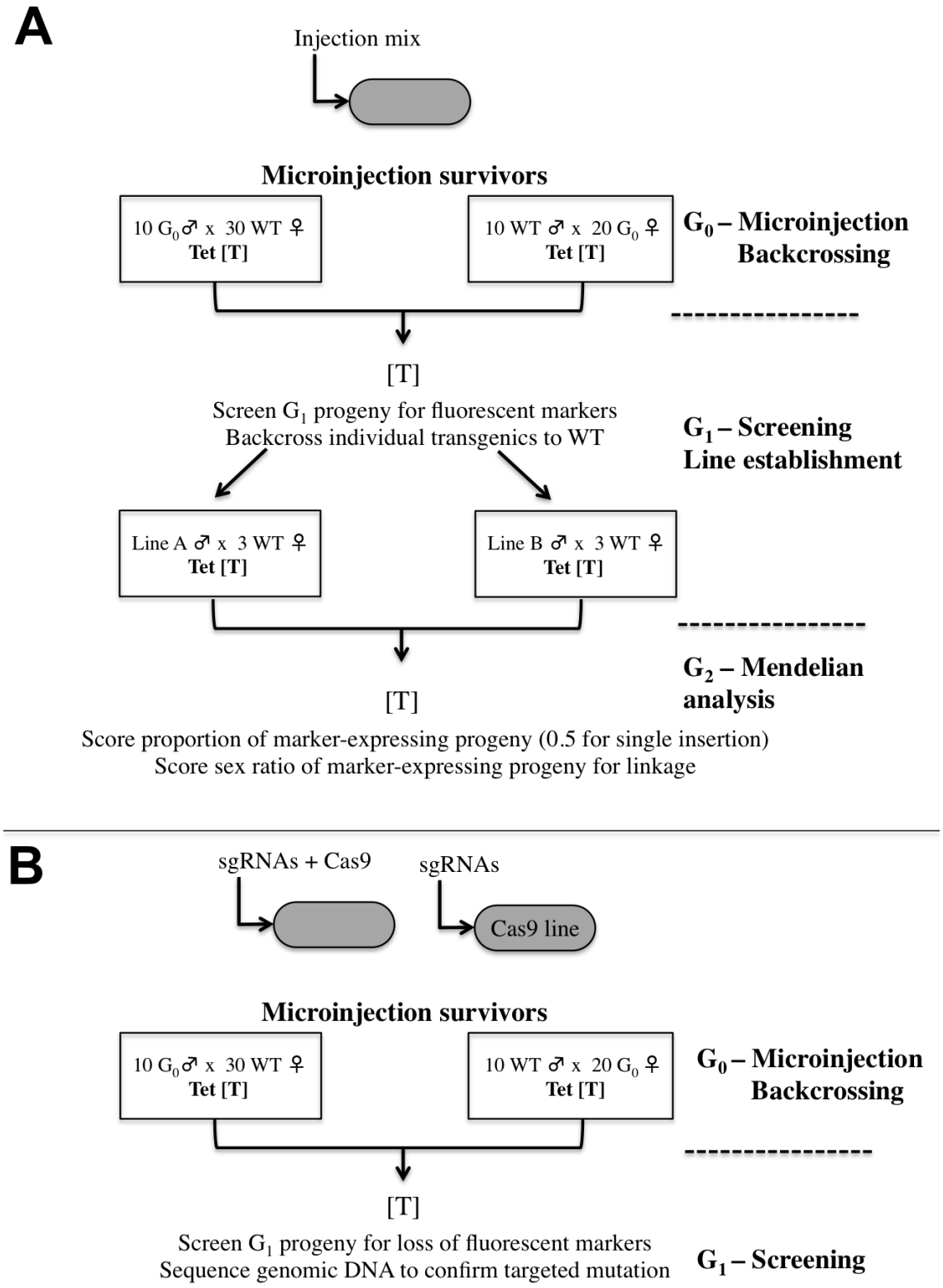
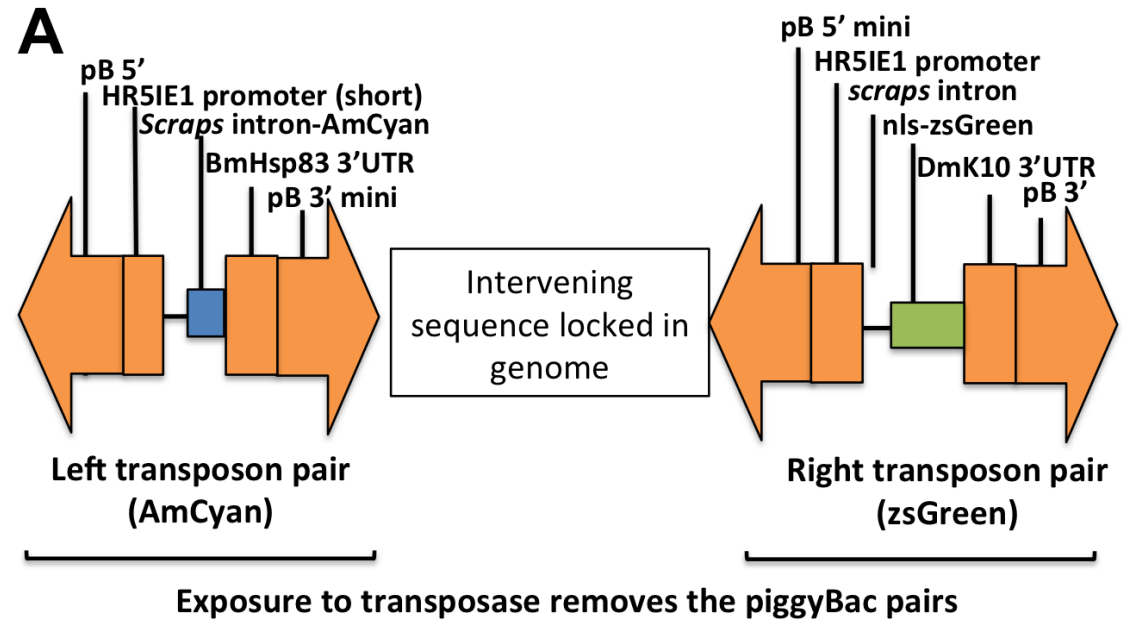


Figure 2.3. Genetic modification with piggyBac or CRISPR. (**A**) piggyBac transgenesis. G_0 individuals were microinjected at the posterior pole with the piggyBac vector and helper (transposase). Survivors were backcrossed to WT. G_1 progeny were screened for transgenesis by expression of the fluorescent marker. Single individuals were independently backcrossed to WT, and the inheritance of the marker monitored to assess transgenic copy number and sex-linkage. (**B**) Example of genetic modification with CRISPR: targeted mutagenesis of a fluorescent marker sequence. Target-specific sgRNAs are injected into embryos (either with Cas9 recombinant protein, or into a transgenic line that expresses Cas9). Progeny are screened for the loss of the fluorescent marker (in this example, DsRed2).

2.3.4 Genomic immobilisation of four-ended *piggyBac* transposons

The sequences required for the transposition of *piggyBac* vectors can be removed with the four-ended *piggyBac* vector system, locking the construct at the genomic insertion site (Dafa'alla et al., 2006). These vectors are composite transposons with two pairs of *piggyBac* ends that self-excise ("resolve"), when crossed to a transgenic line expressing *piggyBac* transposase in the germline (OX3133). Thereafter, the *piggyBac* end pairs transpose away from the central element with the transgenic effectors, leaving it immobile. Fluorescent markers at each *piggyBac* end pair are generally included (eg. OX5195, **Figure 2.2**), to facilitate microscopic screening of *piggyBac* end excision (by marker loss). Subsequent backcrossing to WT generates individuals lacking the *piggyBac* ends but retaining the central element of the construct to be immobilised. The excision is thereafter verified by PCR (absence of the *piggyBac* end sequences) and sequencing across the junction of genomic DNA and expression construct. It is possible that the central element can transpose to a new insertion site during the immobilisation process, potentially altering the phenotype. Therefore, the male sterility phenotype should be reassessed prior to establishing a homozygous strain. The process is summarised in **Figure 2.4**.



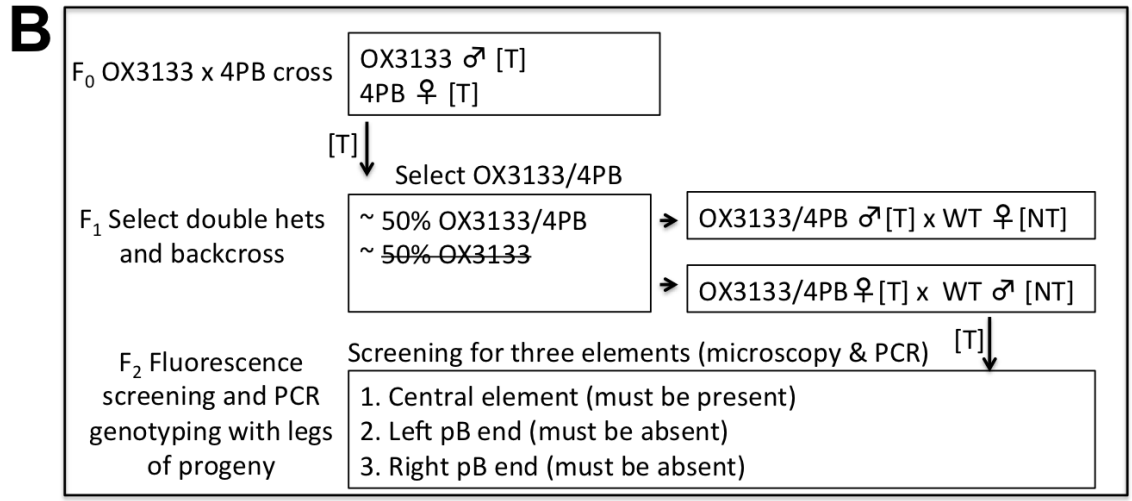


Figure 2.4. Removal of the piggyBac ends from 4-ended vectors (resolution). (**A**) Typical construction of a four-ended piggyBac vector. When exposed to transposase, the left and right piggyBac pairs transposase elsewhere in the genome, immobilising the central element encoding the transgenic effectors. (**B**) Crosses performed to immobilise the transposon. Heterozygous transgenic individuals with the four-ended piggyBac insertion are crossed to OX3133 (the transposase source). F_1 progeny carrying both constructs are selected by fluorescent microscopy and backcrossed to WT. F_2 progeny are screened by fluorescence microscopy for the absence of translation of markers associated with the piggyBac ends (usually zsGreen and AmCyan), but the presence of the marker associated with central element of the construct (dsRed2). The presence or absence of these sequences is thereafter verified by PCR and sequencing is performed across the junction of genomic DNA and the transgenic expression construct. All individuals are reared with tetracycline (T), with the exception of the WT individuals used for backcrossing.

2.3.5 Establishment of homozygous transgenic strains

2.3.5.1 Single pair crosses

Single pairs were randomly crossed from a pool containing a mixture of homozygotes and heterozygotes; the lack of WT progeny in subsequent generations of sibling crosses confirms that the colony is homozygous (**Figure 2.5**). First, the transgenic allele was enriched in the population by crossing heterozygotes for one or two generations (F_0). After removing WT individuals, which do not express the transgenic marker, the population is approximately 1/3 homozygous and 2/3 heterozygous (if there are no fitness penalties associated with homozygosity). Single pairs of F_1 individuals randomly selected from this pool were crossed in small cages (usually 20 pairs). The proportion of single pair crosses yielding a homozygous colony is 1/9 (assuming no fitness penalties). F_2 progeny were screened to eliminate crosses of two heterozygotes (25% of progeny of these crosses are WT). Thereafter, homozygous colonies were differentiated from colonies originating from one homozygous and one heterozygous parent. F_2 progeny of the remaining colonies (siblings) were independently crossed in medium cages for two more generations ($n > 100$). Homozygosity is indicated by absence of WT progeny in the F_3 and F_4 generations. The strategy is time-effective and does not require molecular analysis. However, it can reduce fitness because it constrains the genetic diversity of the colony to two individuals. It was suitable for certain applications, such as the generation of a homozygous fluorescent marker expressing strain (OX4014) for CRISPR method development (Chapter 6). However, it cannot be used for development of homozygous strains to be used in the field for population control, as the risk of a fitness penalty is too high.

2.3.5.2 PCR genotyping of homozygotes

For applications requiring a genetically diverse population, PCR genotyping is necessary to establish a genetically diverse homozygous colony (ideally, originating from at least 50 individuals). However, this requires the genomic sequences flanking the insertion, to differentiate heterozygotes and homozygotes. As previously described, genomic flanking sequence may be identified by inverse PCR or related techniques. To perform homozygous genotyping by PCR, two reactions are required. The first amplifies genomic sequence upstream and downstream of the transgenic insertion ("flanking-specific"). The intervening transposon is too long to be amplified; hence the reaction only amplifies the WT allele, and therefore distinguishes WT and heterozygous

transgenics from homozygous transgenics. The second reaction specifically amplifies the insertion allele, as the primers are designed against genomic sequence and immediately adjacent construct sequence ("transgene-specific"). This distinguishes WT from heterozygous or homozygous transgenic individuals. Therefore, PCR on homozygous individuals generates products for the second reaction, but not the first. However, the genomic sequences of the region containing the genomic insertion site may vary within the population. This can lead to a false indication of homozygosis (the flanking-specific reaction might fail, in both heterozygous and homozygous individuals). Therefore, it is necessary to genotype a large number of WT or heterozygous individuals with the flanking-specific PCR reaction, to confirm that an amplicon is generated in all instances. This was usually performed on 90 individuals.

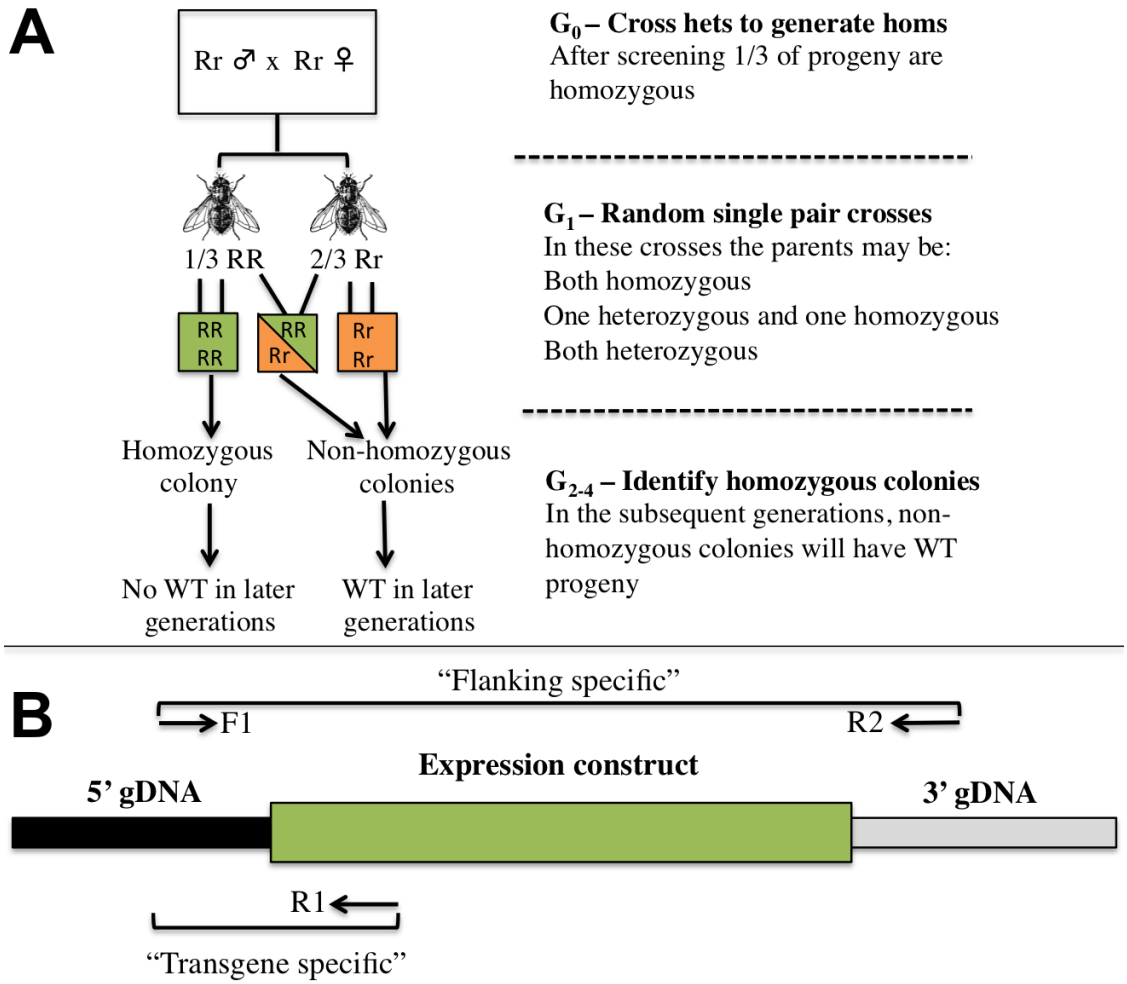


Figure 2.5. Two methods to generate homozygous transgenic lines. (A) Single pairs are randomly crossed from a pool with heterozygotes (Rr) and homozygotes (RR). The homozygous colonies originating from a homozygous father and homozygous mother are differentiated from all other crosses, because the subsequent generations do not have WT progeny. (**B**) For PCR genotyping, two PCR reactions are performed. The first ("flanking-specific"; primers F1 & R2) does not give a detectable product in homozygotes (the intervening transposon is too large to amplify). The second ("transgene-specific"; primers F1 & R1) gives a product in homozygotes and heterozygotes, but not WT. Homozygosity is indicated by an unsuccessful first reaction and a successful second reaction.

2.4 Microdissection and microscopy of the male and female reproductive tract

Flies were anaesthetised on ice or within a chest freezer prior to dissection, for immediate microscopic analysis (**Figure 2.6**) or downstream molecular biology. Care was taken to avoid over-exposure of females to cold temperatures; prolonged exposure causes female *D. melanogaster* to expel sperm (Ashburner et al., 2011). Dissection was performed in PBS (Sigma P4417) or testis buffer (187 mM KCl, 47 mM NaCl, 10 mM Tris pH 6.8), under a stereoscope and lamp (Olympus SZH10 and Photonic PL2000, or similar). The anaesthetised fly was transferred to a single drop of buffer on a microscope slide or plastic plate. The body was firmly held with a pair of tweezers and the male or female genital tract carefully removed by pulling the posterior with a fine pair of tweezers. For DNA extraction, samples were preserved in PBS or testis buffer at -20° C. For RNA extraction, samples were stored in lysis buffer (Norgen Total RNA kit) at -80° C. To image the cells present in the male germline or the presence of sperm in the female reproductive tract, the dissected testes or female reproductive tract were gently squashed under a cover slip and observed at Cardiff University (Olympus BX50 microscope, Hamamatsu Orca 05G Digital Camera, and Olympus U-RFL-T Fluorescence Lamp) or Oxitec (Motic BA210 microscope, Fraen fluorescence FLUOLED lamp, Lumenera Infinity 2 camera). Generally, for testis dissections, 20x and 40x magnification were used; and for the female reproductive tract, 10x and 20x.

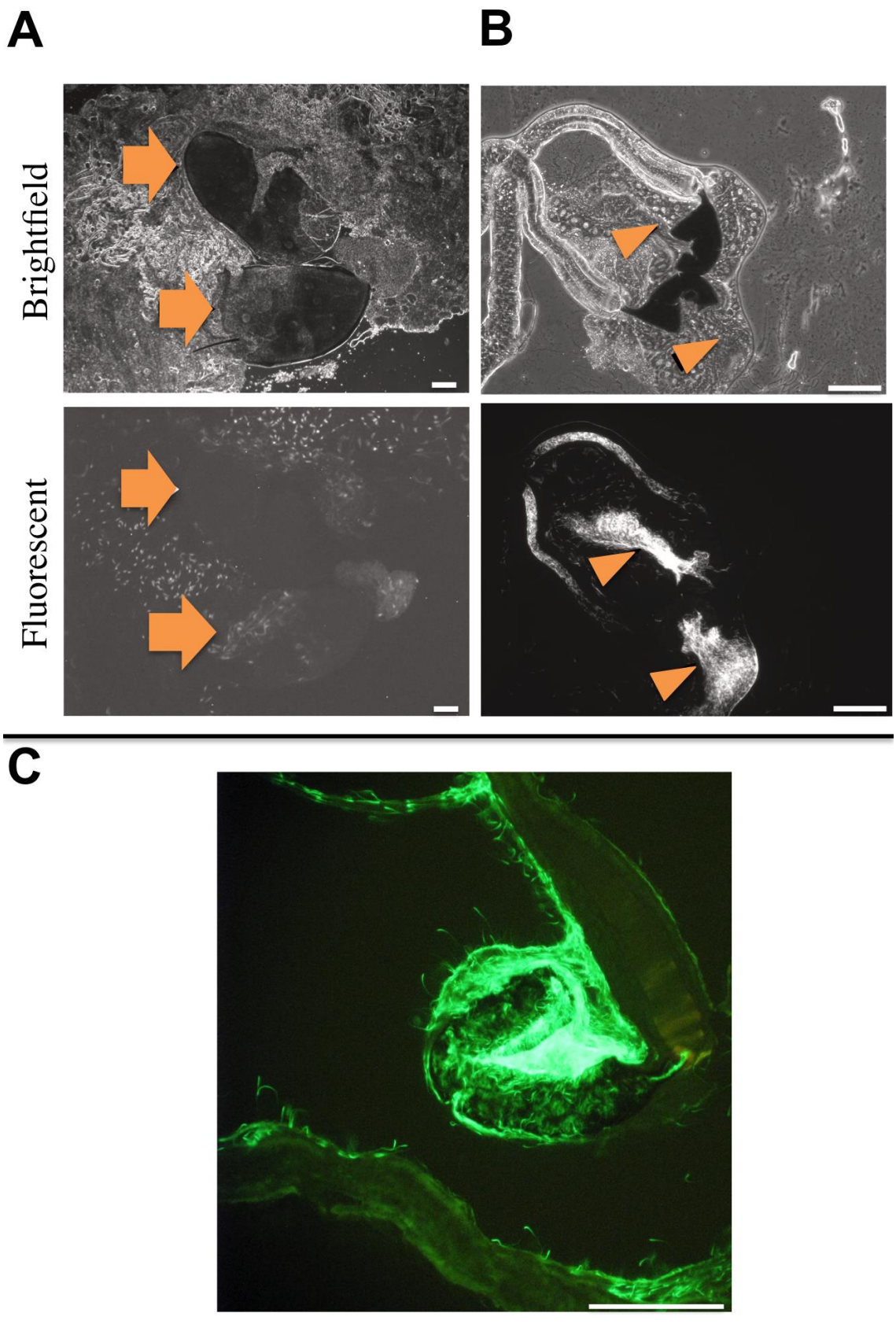


Figure 2.6. Visualisation of fluorescently marked sperm (Ccprot1-zsGreen) in the male and female reproductive tracts. (A) Fluorescence and phase contrast microscopy of dissected Medfly testes (OX5122G). Testes indicated with block arrows. (**B**) Fluorescence and phase contrast microscopy of dissected spermathecae of a female mated to a transgenic male (OX5242(1)H1). Fluorescent sperm indicated with arrowheads. (**C**) True colour micrograph of OX5122M homozygous sperm in the spermathecae of a WT female. Sperm are visible in the spermatchecae and oviducts. Scale bars: 100 μm.

2.5 Phenotypic analysis of expression systems for fluorescent sperm marking

2.5.1 Analysis of fluorescent marker localisation in the male germline

These experiments were performed to assess the performance of transgenic systems for the fluorescent marking of sperm nuclei (**Chapters 3-5**), or to evaluate the ability of regulatory sequences to appropriately regulate translation of a fluorescent reporter in the male germline (**Chapter 4**). Transgenic males were collected < 8 hours post-eclosion (day 0) and kept virgin in small cages (10-20 individuals per cage). Testes were dissected and imaged as previously described, at Cardiff University or at Oxitec. Five to twenty males per line were assessed (generally ten). Localisation of the fluorescent marker in spermatocytes, spermatids and sperm was investigated.

2.5.2 Assessing the presence of fluorescently marked sperm in the female reproductive tract

It was necessary to confirm that the expression of transgenic effectors for repressible male sterility or fluorescent marking of sperm nuclei, were not associated with effects on the morphology, motility, or number of sperm transferred to WT females after mating. These studies were performed either in parallel with an assessment of the mating competitiveness of males (**Section 2.6.2**), or independently. When performed independently, crosses of transgenic males to WT females were performed in small cages. Several studies were performed; details of the crosses are provided in the relevant results section. Between days 5-7 after the cross was initiated, females were removed from the cage after careful anaesthetisation in a chest freezer (-20° C), until females were unable to fly but still able to walk (about 10 minutes).

When performed in parallel with mating competition assays (**Section 2.6.2**), mating pairs were captured immediately in mating chambers (30 ml plastic tubes with airholes), allowed to copulate until dissociation, and the male was removed. Food was added to the tube and the females assessed 24-48 hours later, after immobilizing them by crushing the head. In all studies, the reproductive tract was removed by microdissection and imaged as previously described, at Cardiff University or Oxitec.

2.5.3 Fluorescence microscopy assay to distinguish females that had mated WT males or transgenic males

To study the detectability of fluorescently marked sperm in the female reproductive tract under field-simulated trapping conditions, and therefore differentiate whether females had mated WT or transgenic males, the following test was performed. WT females (n = 100) were independently crossed to transgenic males (n = 100) or WT males (n = 100) in single large cages; mating was allowed for 5 days. To control for fitness differences resulting from rearing density, flies were reared with equal cohorts (n = 700 eggs per bottle). Females were removed from the cages by careful anaesthetisation in the freezer, as previously described. 20 females from each group (mated to WT or transgenic males) were affixed to yellow sticky traps (Seabright Laboratories) for 0, 1, 7, or 14 days prior to scoring; this was done to simulate the process of monitoring released insects in the field (Harris et al., 1971). Double blind scoring was applied to preclude experimenter bias (the genotype of the male was not known prior to scoring). Flies were detached from the trap with tweezers and placed into tubes with distilled water and rinsed by inverting the tube. The abdomen was excised and spermathecae dissected in PBS, prior to gentle squashing under a cover slip and imaging. Frequently, the aged samples (7-14 days) were heavily degraded (due to decomposition after dying on the trap), preventing specific dissection of intact spermathecae. In these instances, the entire abdominal tagma was crushed under a cover slip and imaged as described previously. At each timepoint, we scored the number of individuals for which the male genotype was correctly identified; misidentified as WT (false negatives); or misidentified as transgenic (false positives).

2.6 Phenotypic analysis of transgenic expression systems for repressible male sterility (tetO-protamine-FokI)

2.6.1 Egg hatch rate assays

The rates of egg hatching in the progeny of males reared with tetracycline [T] and without tetracycline [NT] were compared, to evaluate the penetrance and repressibility of the male sterility phenotype conferred by the tetO-protamine-FokI effector (**Figure 2.7**). Transgenic males reared off tetracycline should have a paternal effect lethal phenotype if the line is penetrant, wherein the hatch rate of progeny is minimal or zero. Conversely, the progeny of transgenic males reared on tetracycline should hatch at a rate similar, and ideally equivalent, to the WT control.

In the generation before the assay, two F_0 crosses were performed. In the first, males or females heterozygous for the expression construct were backcrossed to WT in a small or medium cage (to amplify the population of heterozygous males for further experimentation). Transgenic males were generally used, but in some instances, this was not possible (certain strains were irrepressibly male sterile). In the second, WT was reared in extra large cages under standard conditions, for use as a control in the study. Eggs from both crosses were independently filtered onto media with tetracycline [T] or without tetracycline [NT]. WT and transgenic males of the F_1 generation, reared with and without tetracycline, were independently collected on eclosion. Thereafter, each group was crossed in small or medium cages to WT females reared off tetracycline. There were four experimental groups, comprising the two male genotypes, reared with and without tetracycline (**Table 2.6**).

Table 2.6 Crosses performed in the egg hatch rate assay

Group	**Male**		**Female**		**Expected result**
	Genotype	**Tetracycline**	**Genotype**	**Tetracycline**	
1	Transgenic	NT	WT	NT	Low hatch rate
2		T			Normal hatch rate
3	WT	NT			
4		T			

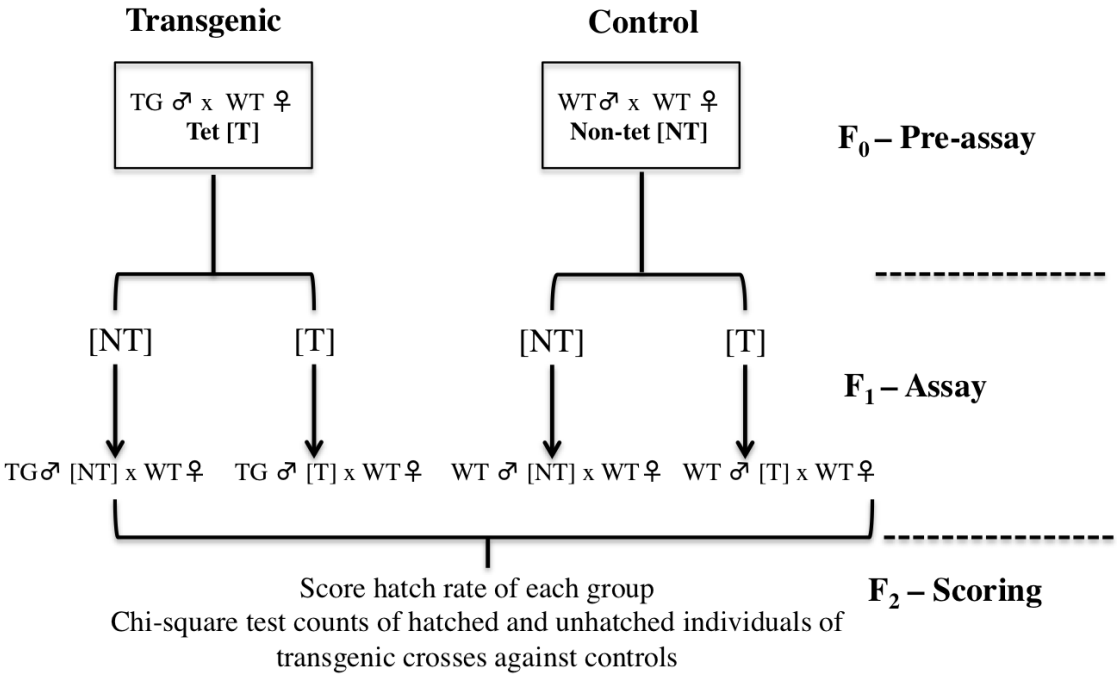


Figure 2.7. Egg hatch assay method. In the pre-assay generation (F_0), the transgenic (TG) line and WT are reared independently under standard conditions. F_1 progeny of both genotypes are filtered to media with tetracycline [T] and without [NT]. Four groups of males are crossed to WT [NT] females: transgenic [NT]; transgenic [T]; WT [NT]; and WT [T]. The hatch rate of the progeny of each group are compared to assess the penetrance and repressibility of male sterility. Progeny of transgenic [NT] males should not develop.

The laying pots were cleared the day before the assay, to facilitate a 24 hour collection of 100-300 eggs, usually on day 7. Eggs were passed through a coarse sieve to remove water from the collection tray, and transferred with a fine paintbrush or Pasteur pipette to a moist, gridded Whatman grade 3 filter paper (Sigma-Aldrich Z240427) under a stereoscope. This was placed in a hatching chamber (an inverted Petri dish sealed with Parafilm) and the hatch rate scored four or five days later. For preliminary tests to identify lines with commercially suitable phenotypes, measurements were pseudoreplicated (collections were taken from the same cage), two or three times. The rationale was that it was unnecessary to conduct an overpowered study at this stage, because most lines would not demonstrate a suitable male sterility phenotype (Jin, 2011), (Asadi, 2013). For lines that demonstrated a commercially promising phenotype, the study was repeated with true replication (four collections from independent crosses). The details of the crosses and collections performed are provided in the relevant results section.

Replicates were tested by correction-free chi-square testing for significant variance, prior to pooling individual replicates. Penetrance and repressibility were calculated based on the mean hatch rates for the transgenic [NT] and [T] crosses, relative to the WT [NT] and WT [T] controls. Formulae are provided below. The statistical significance of the penetrance of male sterility was calculated by correction-free chi-square testing of the number of hatched and unhatched individuals of the transgenic [NT] cross, against the WT [NT] control. The statistical significance of the repressibility of male sterility was calculated equivalently against the WT [T] control, in the instances where this control was performed. For lines that appeared to be non-repressible, the extent of repressibility was assessed by correction-free chi square testing of the [NT] and [T] hatch rates for each line. Standard error and 95% Wilson confidence interval (CI) were calculated by the methods recommended for categorical data (Xu et al., 2010).

$$Penetrance = 100 - \left(\frac{Transgenic[NT]\ hatch\ rate}{WT[NT]\ hatch\ rate} * 100\right)$$

$$Repressibility = \frac{Transgenic[T]\ hatch\ rate}{WT[T]\ hatch\ rate} * 100$$

2.6.2 Lab-scale mating competition assays

These tests were performed according to IAEA guidelines (FAO et al., 2003) to assess if transgenic, conditionally sterile males could compete equally with wild-type males for mates (**Figure 2.8**). These tests are only expected to highlight severe fitness defects because they are performed in an enclosed space at high population densities. Furthermore, mating behaviour observed in a laboratory environment does not accurately reflect wild behaviour (Liimatainen et al., 1997), (Briceño and Eberhard, 1998). Hence, it is therefore necessary to perform further testing under conditions that more effectively simulate wild conditions, such as greenhouses, preferably with wild males (rather than WT).

We only assessed WT and transgenic males that had been reared off tetracycline (NT). If these transgenically sterilised males were able to compete adequately, it would obviate the need to assess the performance of tetracycline-reared males, which would be expected to express the male sterility effector to a lesser extent (it would be repressed). Furthermore, the mating competitiveness of tet-reared males was relatively unimportant,

because tet-reared males will not be released into the field. Rears were quality controlled by weighing young pupae on an Ohaus Pioneer Analytical Balance PA64 (< 24 hours post-pupation); rears with a mean pupal mass under 7 mg were rejected (FAO et al., 2003). Upon eclosion, WT males, transgenic males and WT females were collected as virgins and reared as separate groups, in small cages with 25 individuals. The assay was performed five days after eclosion in the morning (initiated between 08:00-10:00), in Bugdorm cages with equal numbers of transgenic males, WT males and WT virgin females (n = 50 each).

Males of both genotypes were released into the Bugdorm cage and left for one hour to establish territory. Wild-type females were then added; mating usually begins within five minutes. Any females that had not attempted mating within one hour were excluded from the analysis. After mating pairs stabilised (about 5 minutes), they were trapped within a mating chamber (a 30 ml universal tube with airholes), allowed to walk up the side, and the chamber sealed. The chambers were placed atop a tray and the tubes observed every 10 minutes, to record the duration of mating. Pairs mating for less than one hour were discarded. The genotype of the male was scored (by the presence or absence of the AlMAct-dsRed2 transformation marker). Three or five replicates were performed, and the observations pooled after verifying that there were no significant differences between the replicates, by chi-square testing. Differences in mating duration between WT and transgenic males were assessed by independent sample, two-tailed t-test. The relative sterility index (RSI), a metric of competitiveness defining the proportion of females paired with transgenic males (N_{tg}) relative to total mating events ($N_{tg} + N_{wt}$), was calculated (Cayol et al., 1999):

$$\mathbf{RSI} = \frac{N_{tg}}{N_{tg} + N_{wt}}$$

Therefore, the RSI value indicates whether, under these conditions, transgenic males are more competitive relative to WT (RSI > 0.5), less competitive (RSI < 0.5), or equally competitive (RSI = 0.5), for mating events with females.

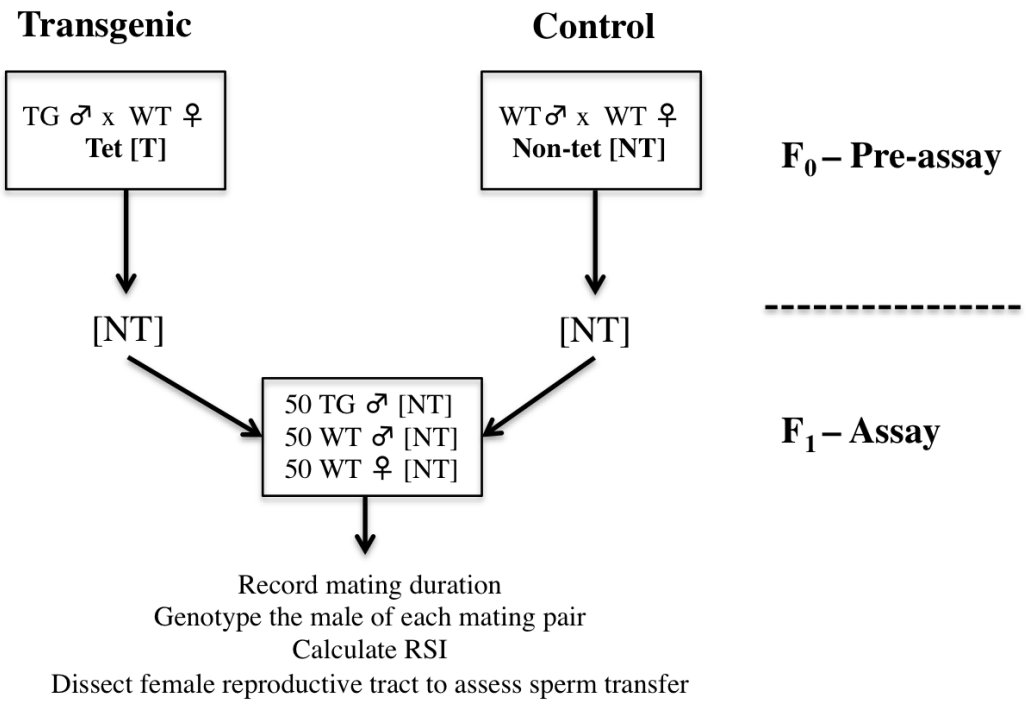


Figure 2.8. Mating competition assay method. In the pre-assay generation (F_0), the transgenic (TG) line and WT are reared independently under standard conditions. F_1 progeny of both genotypes were filtered to media without tetracycline [NT]. Males of both genotypes were placed in a single Bugdorm cage with WT females, and the number of mating events of females to each male genotype compared (RSI). An RSI of 0.5 is ideal.

Assistance from colleagues was provided for both of the two strains assessed in this study (OX4718A and OX5122M). Three or five replicates were performed per study. For each replicate, one individual collected the mating pairs from a single cage over the course of one hour. The author conducted one replicate; the other replicates were assessed in parallel by colleagues (Thea Marubbi, Ben Granville, Charilaos Megas or Christa Kistenpfennig). The subsequent data collection and processing (genotyping the males, recording the mating duration, post-collection dissections, and data analysis) were conducted by the author.

2.6.3 Visualisation of the paternal effect lethal phenotype by nuclear staining of embryos

Expression systems for male sterility should arrest the development of all embryonic progeny of off-tet reared males (NT), for both heterozygous and homozygous transgenic lines. However, it is likely that the effect will be stronger in homozygotes, because of increased transgene expression from the presence of a second copy of the effector. The crosses performed were essentially as described for the egg hatch assay. Off-tet and on-tet reared transgenic males were crossed independently to off-tet reared WT females. Controls were equivalent crosses with WT males (non-tet and tet-reared) and WT females. Therefore, there were four experimental groups (**Table 2.7**).

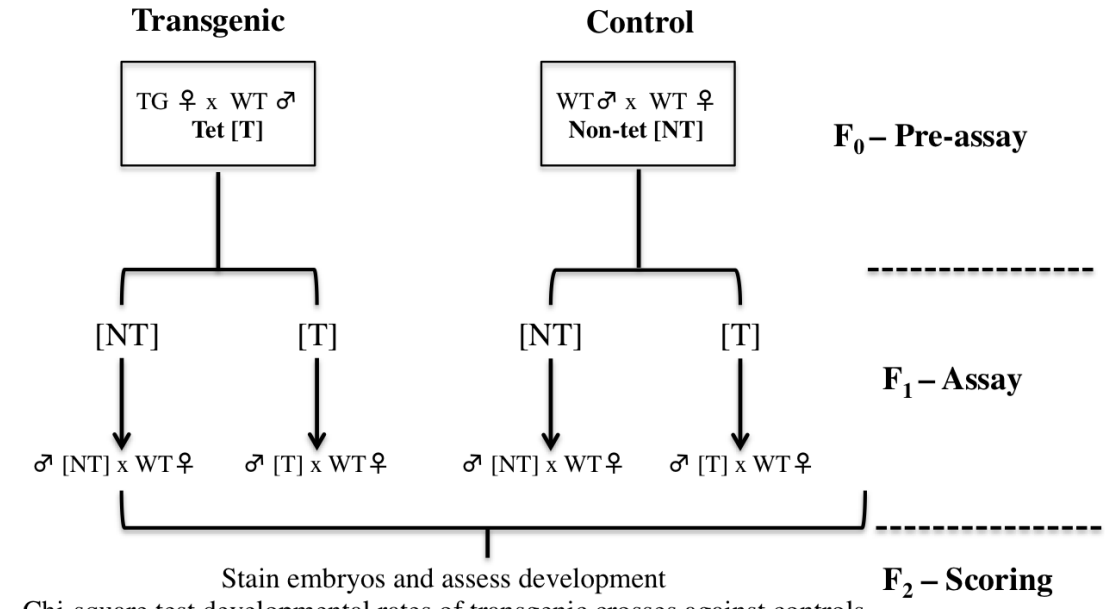
Table 2.7 Experimental groups for the embryonic nuclear staining assay

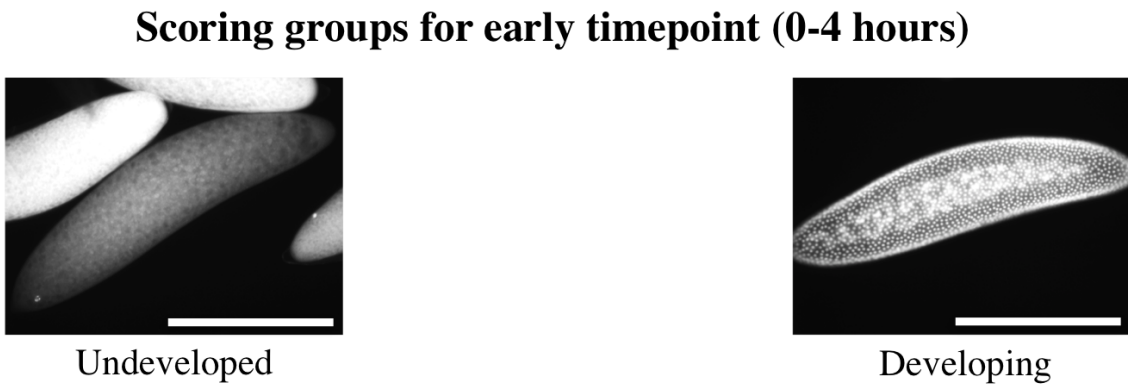
Group	Male		Female		Expected result
	Genotype	**Tetracycline**	**Genotype**	**Tetracycline**	
1	Transgenic	NT	WT	NT	Minimal development
2		T			Normal development
3	WT	NT			
4		T			

Eggs were collected from each cross in four hour cycles, fixed and stained (days 7-9). Two timepoints were assessed: 0-4 hours and 20-24 hours after oviposition. To fix, embryos were dechorionated with 10% sodium hypochlorite solution (2 minutes) and rinsed with water to remove bleach, essentially as described for microinjection. Dechorionated embryos were transferred with a fine brush to 10 ml glass vials with heptane (5 ml) and 4% paraformaldehyde in PBS (2 ml). To devitellinate and fixate the embryos, they were left in a shaking incubator (room temperature, 2 hours, 250 rpm). The fixative (lower layer) was removed with a micropipette, 5 ml methanol added, and the mixture shaken by hand for 30 seconds. Fixed embryos were transferred to a microfuge tube, rinsed with methanol three times, and all methanol removed thereafter. All subsequent washes and incubations were performed for 15 minutes with a microfuge tube rotator: first, in 50% PBS with 0.1% Tween (50% PBST) and 50% methanol, and thereafter in PBST. Embryos were incubated with RNaseA (0.25 mg/ml) and washed three times with PBST. To stain, incubation was performed with Hoechst 33258 (1 ng/µl), prior to washing with PBST. All buffer was removed and embryos mounted in 85% glycerol with 2.5% n-propyl gallate under a siliconised coverslip, and the edge sealed with clear nail varnish. Embryos were imaged by fluorescence and phase contrast

microscopy (UV filter) at Cardiff University, at 10x magnification with the previously described apparatus (Olympus BX50 microscope, Hamamatsu Orca 05G Digital Camera, and Olympus U-RFL-T Fluorescence Lamp).

At least 10 individuals were scored from each group, for the extent of development. From the 0-4 hour collection, embryos were scored as developing or not developing (several nuclei visible or only first nucleus visible). From the 20-24 hour collection, embryos were scored as morphologically normal, morphologically aberrant, or no visible development (**Figure 2.9**). For statistical analysis, data was divided by timepoint and correction-free chi-square testing was performed with the counts of embryos at each stage, relative to the appropriate WT control ([NT] or [T]). Repression was calculated by equivalent testing of the counts of embryos at each stage, for the [NT] group against the [T] group, for each line. WT was equivalently assessed to control for a effect of tetracycline that affected viability, independently of the male sterility effector.





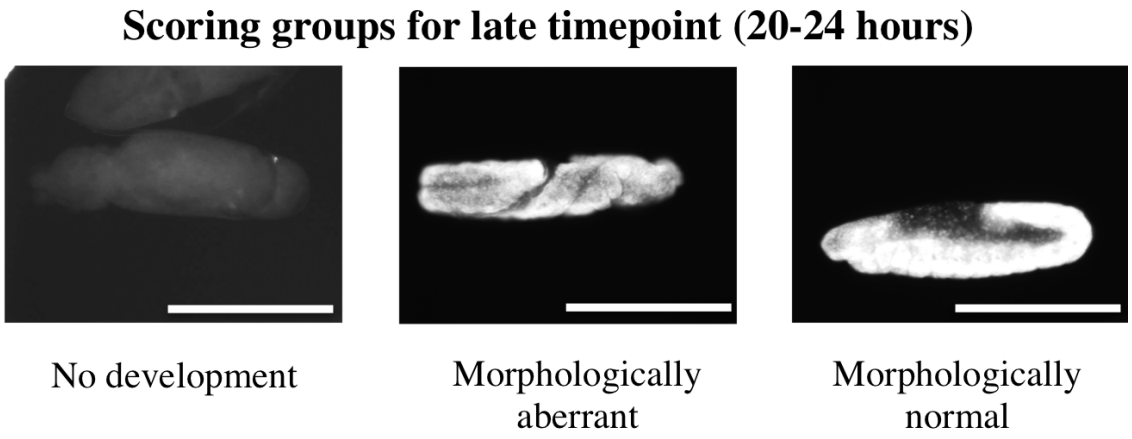


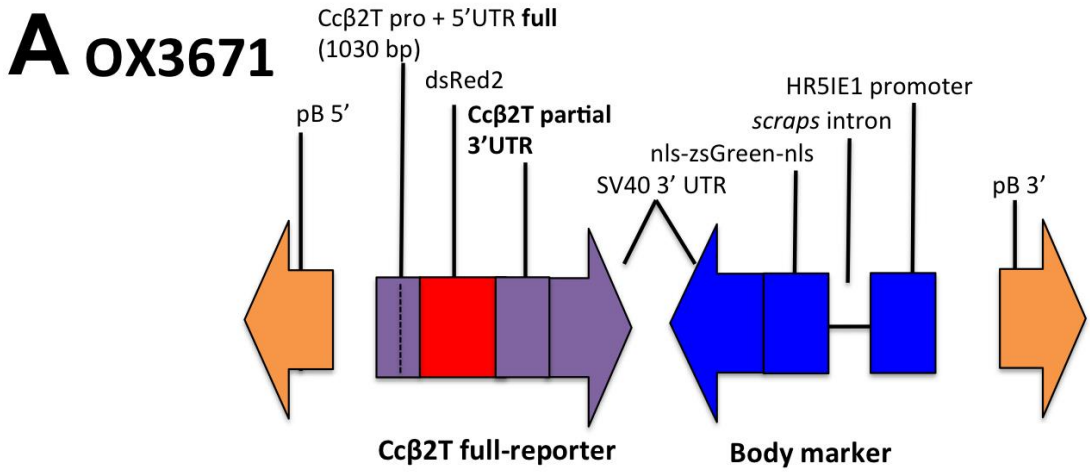
Figure 2.9. Visualisation of embryonic inviability in the progeny of sterile males, by nuclear staining assay. The pre-assay generation (F_0) was generally reared as described for the egg hatch assay. F_1 progeny of both genotypes were filtered to media with tetracycline [T] and without [NT]. Four groups of males were crossed to WT [NT] females: transgenic [NT]; transgenic [T]; WT [NT]; and WT [T]. Embryonic progeny were collected, fixed and stained to monitor development. Minimal or no development is expected in progeny of transgenic [NT] males. From the 0-4 hour collection, embryos were scored as developing or not developing; from the 20-24 hour collection, embryos were scored as morphologically normal, morphologically aberrant, or no visible development. Scale bars: 0.5 mm.

Chapter 3 – Expression systems for fluorescent sperm marking and Dmprot2-FokI mediated repressible male sterility

3.1 Previous development of male sterility and fluorescent sperm marking systems

In prior study (Jin, 2011), a fragment based on the Ccβ2tubulin promoter and 5'UTR (OX3671: Ccβ2tubulin promoter-Ccβ2tubulin 5'UTR[full]-**dsRed2**-SV40 3'UTR) mediated localisation of the dsRed2 fluorescent reporter in spermatocytes, spermatids and sperm, indicating that this fragment would be able to localise tTAV in the male germline, largely as required (**Figure 3.1**). However, translation was not as early as desired (not robustly expressed in all spermatocytes). High levels of pre-meiotic transcription and translation of tTAV in the male germline were required for sufficient transcriptional activation of the protamine-FokI male sterility effector, at the target tetO sites. The practical reasons for this were fully outlined previously (**Section 1.4.2.3**). Briefly, it is necessary for adequate protamine-FokI transcript to accumulate prior to meiosis, because transcription is highly reduced in the male germline after this point. However, the translation of protamine-FokI must be delayed until spermatid elongation, to avoid disruption of the meiotic divisions, or defects in spermatid nuclear shaping that might prevent spermatid individualisation.

To achieve an earlier translational profile, the Ccβ2tubulin 5'UTR was truncated to remove putative elements that could delay translation, and attached to a minimal promoter fragment and 5'UTR from Cchsp83, a gene with earlier transcription and translation (Jin, 2011). The resulting Ccβ2tubulin-Cchsp83 chimeric fragment (OX4282: tetO14-Ccβ2tubulin promoter-5'UTR[short]-Cchsp83 minipromoter- 5'UTR-**tTAV**-SV40 3'UTR) was found to facilitate appropriately early transcription and translation of a reporter (tetO14-Dmhsp70 minipromoter-*adh* intron-**tGFP**-SV40 3'UTR); it was robustly localised to spermatocytes. This indicated that the fragment would be suitable to regulate appropriately early expression of tTAV. This same chimeric Ccβ2tubulin-Cchsp83 driver was subsequently confirmed to appropriately promote expression of a Dmprot2-FokI effector cloned downstream of the tTAV target sites (tetO), in the male germline (OX4353: **tetO14**-Dmhsp70 minipromoter[+89 bp 5'UTR]-Dmprot2-FokI; **Figure 3.2**). This resulted in penetrant and repressible male sterility (Jin, 2011), (Asadi, 2013).



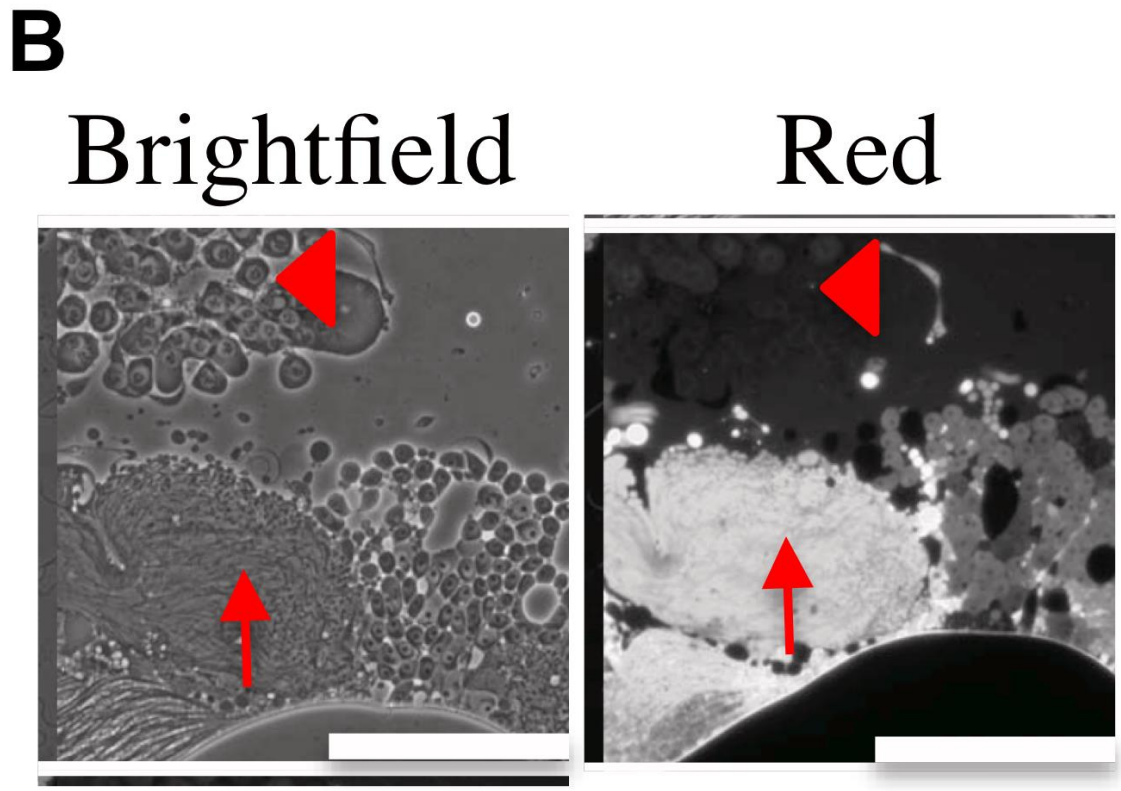


Figure 3.1. Validation of a suitable expression system for tTAV, to engineer repressible male sterility in Medfly (continued on next page). Reporter constructs to test Ccβ2tubulin-derived expression systems (OX3671 & OX4282). (**A**) OX3671 applied a full length Ccβ2tubulin promoter-5'UTR fragment to regulate dsRed2. Dashes indicate promoter-5'UTR boundary. (**B**) Fluorescence and phase contrast microscopy confirmed that it was not translated in all spermatocytes. Annotations: late elongating spermatid (arrow); spermatocytes (arrowhead). Scale bars: 100 μm.

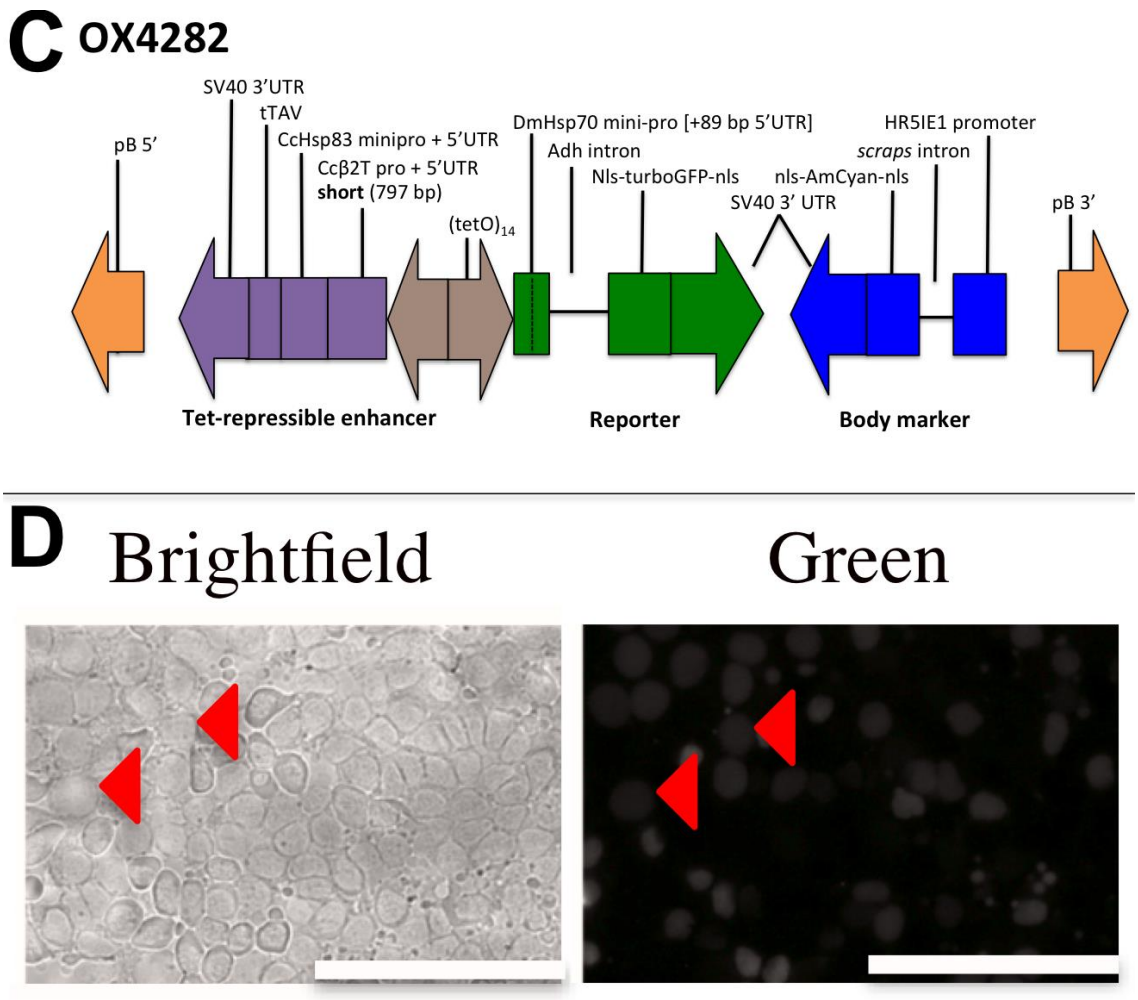


Figure 3.1. Validation of a suitable expression system for tTAV, to engineer repressible male sterility in Medfly. (**C**) OX4282 applied a truncated Ccβ2tubulin 5'UTR fragment fused to Cchsp83 minipromoter-5'UTR. (**D**) This fragment regulated tTAV as desired; the adjacent reporter (tetO14-Dmhsp70-tGFP) was more robustly translated in spermatocytes (arrowheads). However, mature sperm were not adequately marked in either system. Scale bars: 100 μm. **All images adapted from Jin (2011).**

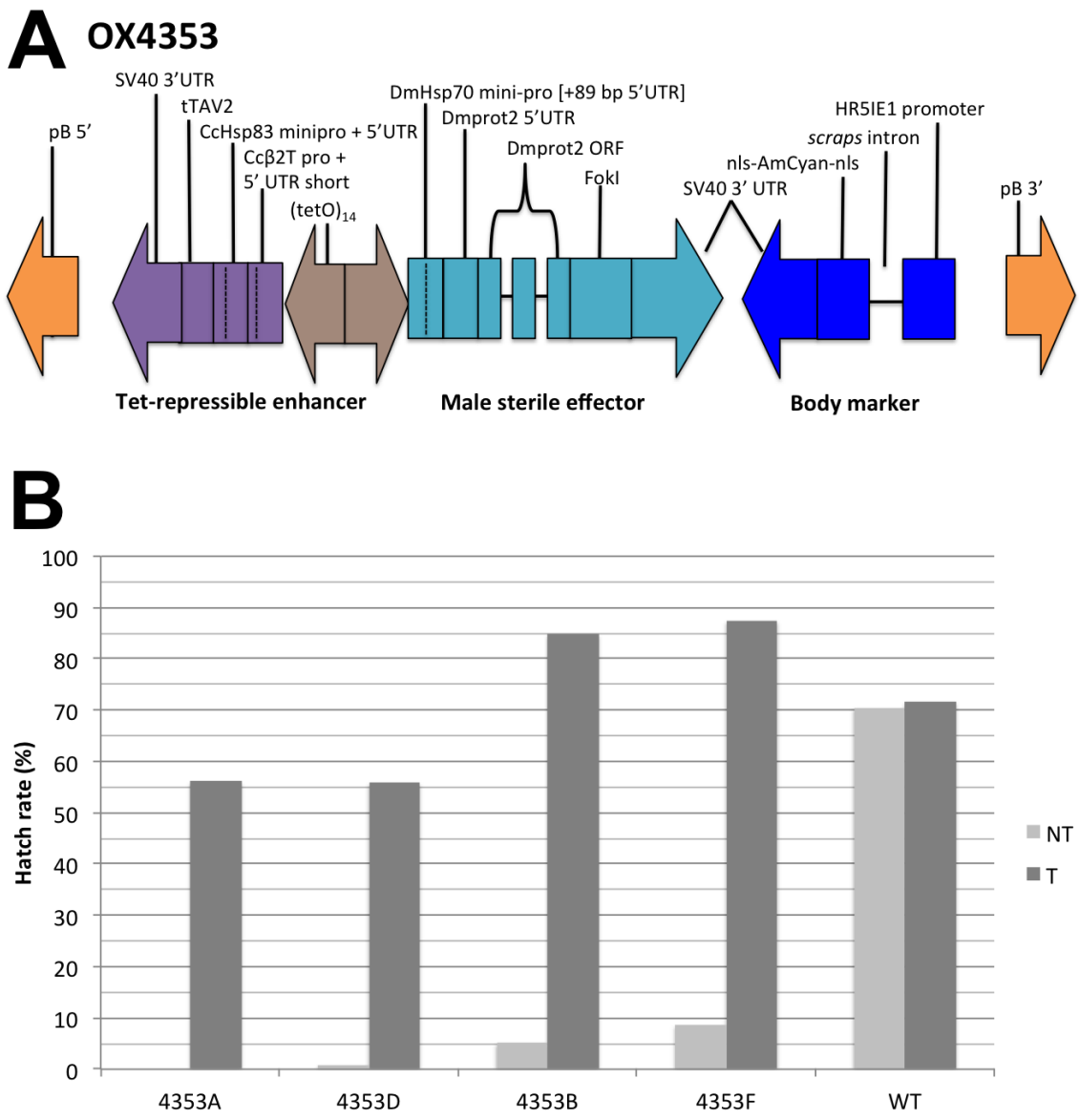


Figure 3.2. OX4353 mediates penetrant-repressible male sterility in Medfly. The tetO14-Dmprot2-FokI effector was placed under tetracycline-repressible regulation with the truncated Ccβ2T-Cchsp83 fragment from OX4282 (see previous figure for construct diagram). (A) OX4353 diagram. Dashes indicate promoter-5'UTR boundary. (B) Egg hatch assay demonstrates penetrant and repressible male sterility (performed by Romisa Asadi). **All images adapted from Asadi (2013).**

Neither reporter construct (OX3671 or OX4282) was functional as a system for fluorescent sperm marking. In OX3671, approximately half of sperm were weakly marked with the dsRed2 reporter. In OX4282, visible marking was observed in only one of five lines. Crucially, neither reporter was associated with a DNA-binding element (for instance, protamine), which would be expected to spatially aggregate fluorescent molecules upon the sperm genome, and hence improve the visibility of the marking system. It was therefore reasoned that a fusion of protamine-fluorescent marker-FokI could simultaneously function as an effector of penetrant and repressible male sterility, and fluorescent sperm marking. However, these constructs did not function as well as anticipated (**Figure 3.3**) because the fluorescent sperm marker and male sterility effector appeared to affect the function of one another (Asadi, 2013). A linker-free fusion of Dmprot2-mCherry-FokI (OX4705) demonstrated penetrant and repressible sterility in two of four olive fly lines, and semi-penetrant sterility in one line. However, fluorescent sperm marking was not observed in any line. Thereafter, it was considered if addition of short flexible peptide linkers (SG4) between the sperm marker and protamine-FokI effector, could improve the phenotype. OX4801 (tetO21-Dmprot2-**SG4 linker**-mCherry-**SG4 linker**-FokI) demonstrated weak but visible sperm marking in all six olive fly lines evaluated, but the penetrance of male sterility was unacceptably reduced (mean hatch rate: 20%). Therefore, if it can be assumed that the effectors function equivalently in olive fly and Medfly, the inclusion of linkers would improve fluorescent sperm marking, but unacceptably reduce penetrance.

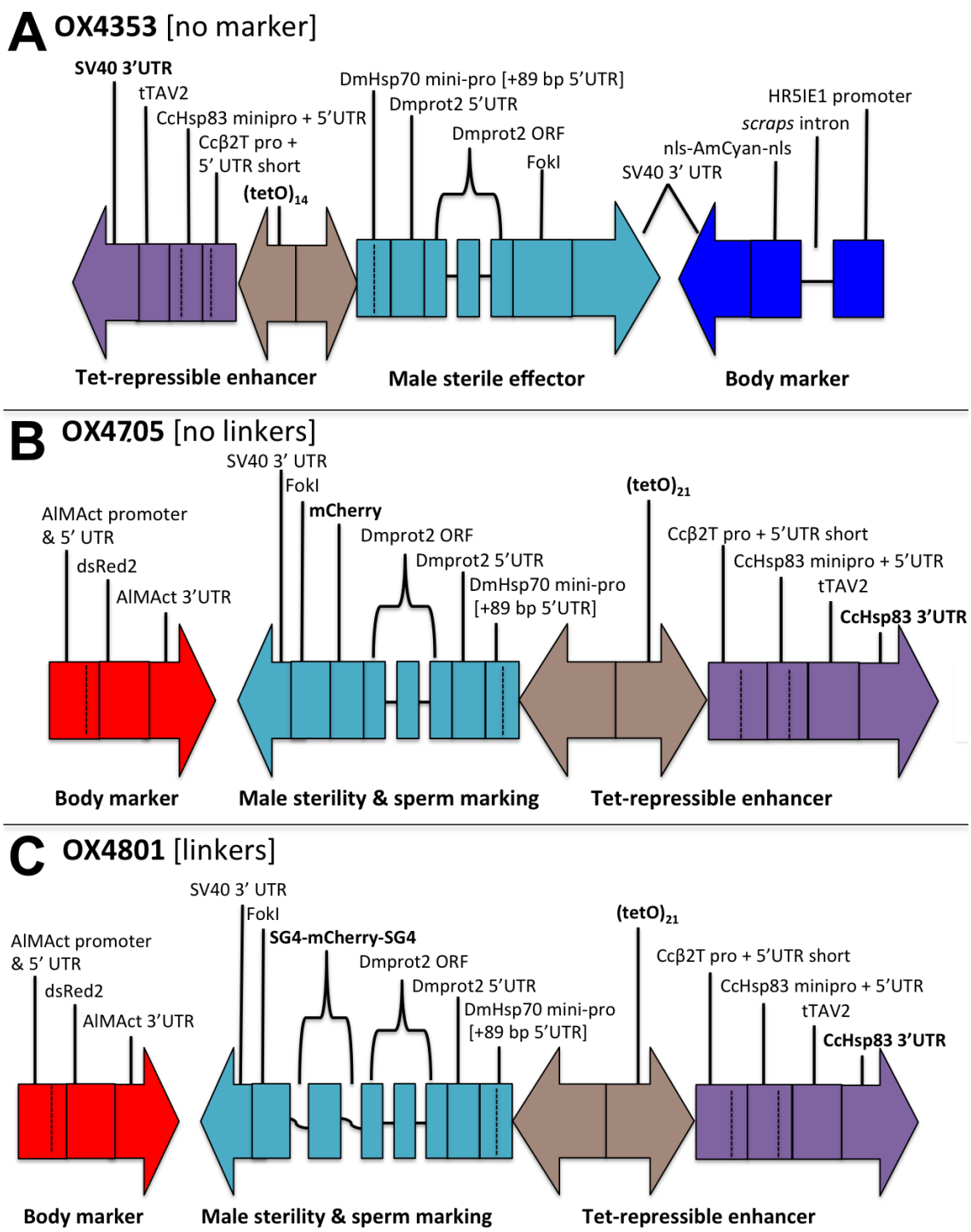
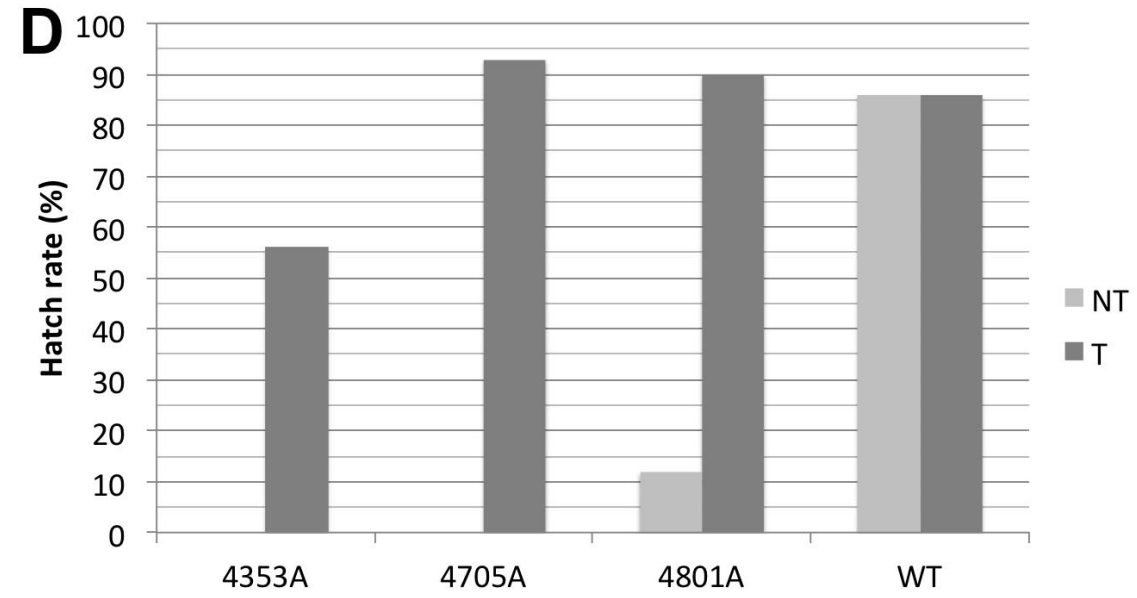


Figure 3.3. Fusions of the male sterility effector and fluorescent sperm marking systems are problematic (continued on next page). (**A-C**) Expression construct diagrams for (**A**) OX4353 (tetO14-Dmprot2-FokI); (**B**) OX4705 (tetO21-Dmprot2-mCherry-FokI [no linkers]) and (**C**) OX4801 (tetO21-Dmprot2-**SG4**-mCherry-**SG4**-FokI). Dashes indicate promoter-5'UTR boundary. Note: piggyBac ends omitted from these two-ended vectors.



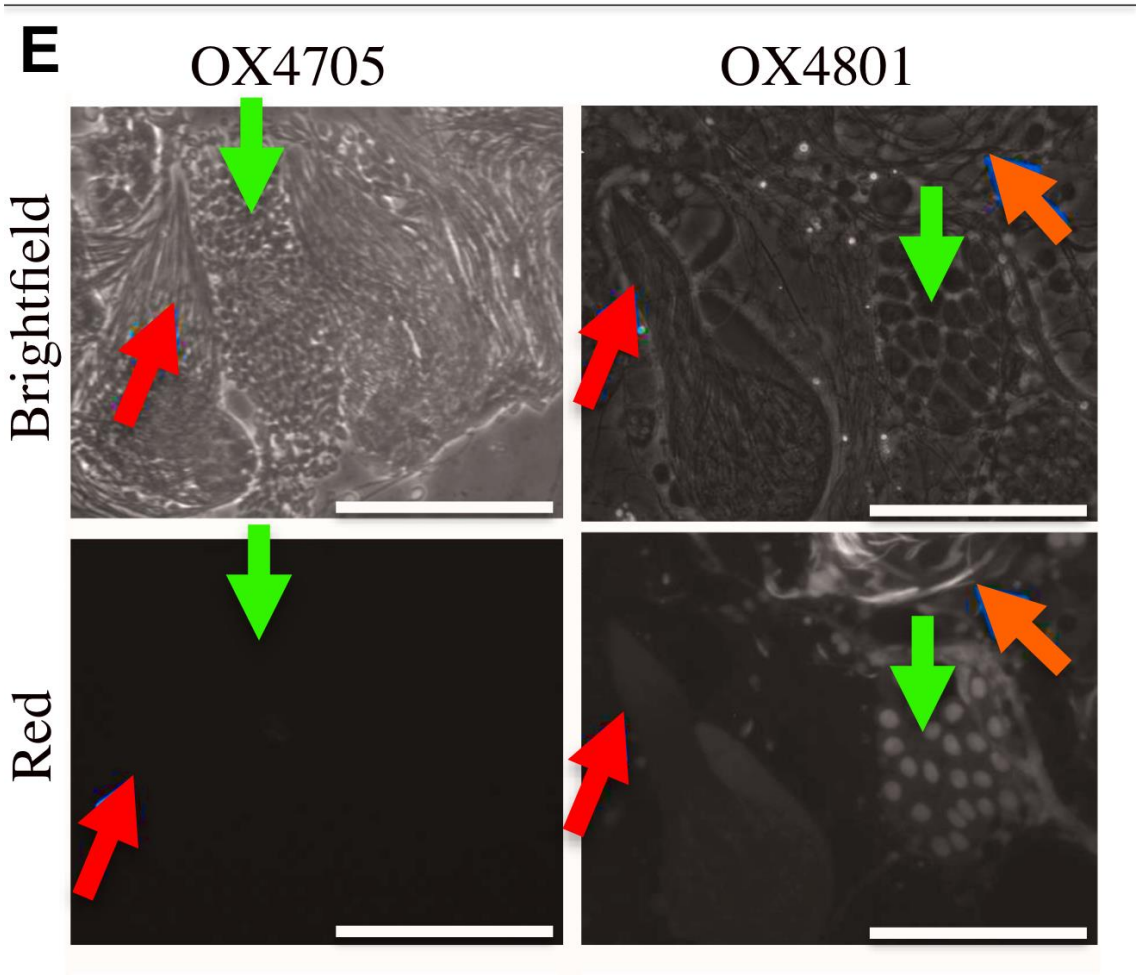


Figure 3.3. Fusions of the male sterility effector and fluorescent sperm marking systems are problematic. (**D**) Investigation of penetrance of male sterility by egg hatch assay in OX4353, OX4705 and OX4801 strains. (**E**) Fluorescence and phase contrast microscopy of marker expression in dissected testes (scale bars: 100 μm). Penetrant and repressible male sterility was observed in constructs that either did not apply a sperm marker (OX4353) or applied a sperm marker without SG4 linkers (OX4705). Sperm marking was, conversely, visible in lines with SG4 linkers (OX4801), but not in lines lacking them (OX4705). OX4353 was evaluated in Medfly; OX4705 and OX4801 were evaluated in olive fly. Annotations: spermatocytes (green arrow); late elongating spermatid (red arrow); individual sperm (orange arrow). **Images adapted from Asadi (2013).**

3.2 Transgenic expression systems for repressible male sterility and fluorescent sperm marking evaluated in this study

We decided to first evaluate the linker-free Dmprot2-mCherry-FokI effector in Medfly, as it was previously shown to mediate penetrant and repressible sterility in olive fly, though the fluorescent sperm marking phenotype was relatively poor. This was cloned into a four-ended *piggyBac* vector (OX4718), which allows excision of the transposable ends after genomic integration (Dafa'alla et al., 2006), preventing subsequent re-transposition (a concern of agencies regulating the use and release of genetically modified organisms). We intended to evaluate the ability of this system to mediate penetrant and repressible sterility, whilst attempting to improve the sperm marking system in a parallel study. We therefore expected to observe penetrant and repressible male sterility with minimal or no marking, if the molecule functioned equivalently in Medfly relative to olive fly, where it was originally characterised. A penetrant and repressible male sterility phenotype was demonstrated for an OX4718 line (Bilski, 2012). However, the functionality of fluorescent sperm marking and the effects of protamine-FokI expression on mating competitiveness, and the appearance and quantity of engineered sperm transferred to females upon mating, were not yet evaluated.

In parallel, we tested a protamine-free sperm marking system in OX5036, which was regulated by tetracycline-repressible, positive feedback expression of tTAV (tetO21-Ccβ2tubulin promoter-5'UTR[short]-Cchsp83 minipromoter-Cchsp83 5'UTR-tTAV-**T2A-nls-zsGreen-nls-**Cchsp83 3'UTR). In the absence of tetracycline, it was anticipated that this fragment would positively regulate its own expression (tTAV-mediated transactivation at the tetO target sequences), leading to high levels of expression. In this design, two polypeptides were translated from the same mRNA (tTAV and nls-zsGreen-nls), separated at the T2A translational skipping element. Translation should occur in primary spermatocytes, and thereafter the nls-zsGreen-nls marker was expected to localise to the nucleus and persist through spermiogenesis, to eventually label the sperm nucleus. It was thought that separation of Dmprot2-FokI from the sperm marker might enhance brightness, because fusions of protamine-marker-FokI were previously demonstrated to mediate poor marking (OX4705), even when separated with linkers (OX4751).

The fragment regulating the male sterility effector in OX5036 (tetO21-Dmhsp70 minipromoter[+89 bp 5'UTR]-Dmprot2 5'UTR-Dmprot2 ORF [no introns]–SG4 linker-

FokI-SV40 3'UTR) was modified from the version in OX4718A. The primary difference was that the mCherry marker between Dmprot2 and FokI was removed. Further changes were made to the effector, in an attempt to enhance penetrance of male sterility (**Figure 3.4**). Introns were removed from the Dmprot2 ORF; the presence of introns can be associated with reduced expression in the male germline (White-Cooper and Caporilli, 2013). The C-terminal end of Dmprot2 was also altered slightly relative to prior constructs. The full WT sequence was included (the C-terminal lysine in OX4718 & OX4353 was removed) and a small tripeptide at the C-terminus was removed. This TMA tripeptide (present in OX4718 & OX4353), was not part of the Dmprot2 coding sequence. Presumably, it had been included in previous constructs to introduce a spacer between Dmprot2 and FokI, which was no longer necessary, because an SG4 linker sequence was included. Neither of these changes were expected to affect localisation of the molecule. It was previously shown that the inclusion of an SG4-mCherry-SG4 fragment (Dmprot2-SG4-mCherry-SG4-FokI) had a negative effect on the penetrance of male sterility. However, it remained possible that a shorter sequence (Dmprot2-SG4-FokI rather than Dmprot2-SG4-mCherry-SG4-FokI) would generate a favourable spatial conformation, and enhance the penetrance of male sterility.

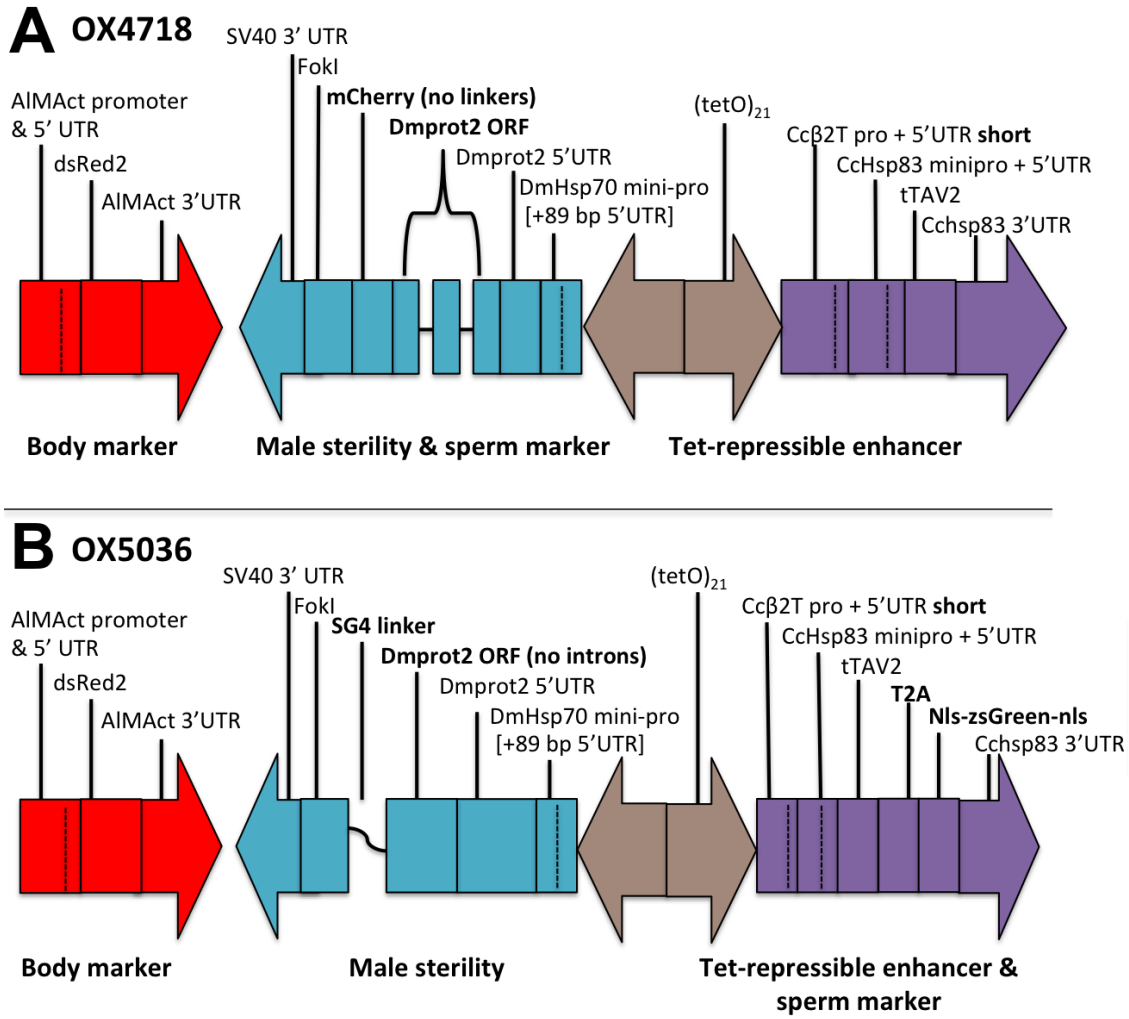


Figure 3.4. Expression constructs for tetracycline-repressible male sterility and fluorescent sperm marking systems. (**A**) OX4718 diagram. (**B**) OX5036 diagram. Dashes indicate promoter-5'UTR boundary. Both feature a bipartite, tetracycline-repressible male sterile system active in the male germline, wherein tTAV transactivates expression of itself and the protamine-FokI effector, at the 21 adjacent tetO sequences. Relative to OX5036, OX4718 was cloned into a four-ended vector; features a fused tetracycline-repressible male sterile system and sperm marking element (without linkers); and the structure of the male sterility effector was changed. Note: piggyBac ends omitted from these vectors (OX4718 is 4-ended and OX5036 is 2-ended).

3.3 OX4718 heterozygous males demonstrate penetrant and repressible sterility but weak fluorescent sperm marking

Two single autosomal insertions (OX4718A & B) were assessed by Michal Bilski prior to this studentship. Penetrance of the male sterility phenotype had been investigated by comparing the egg hatch rate of progeny from OX4718 heterozygous males, reared on- and off-tetracycline, to equivalent control crosses with wild-type males (Bilski, 2012). The assays were initiated on day 4 post-eclosion and scored on day 8. OX4718A appeared highly penetrant (98.8%) and OX4718B largely impenetrant (**Table 3.1**). The hatch rates of progeny of OX4718A males reared on-tet were identical to the equivalent WT control, indicating full phenotypic repression of sterility.

Table 3.1 Penetrance and repressibility of the male sterility phenotype in OX4718

Line	Mean hatch rate (%)		Penetrance (%)	Repressibility (%)
	NT	T		
OX4718A	1.1	92	99	100
OX4718B	81	91	11	100
WT	91	91		

Penetrance and repressibility were calculated relative to the respective control hatch rate, WT [NT] or [T]. Standard error was not calculated (sample sizes were not provided). Note: these experiments were not performed by the author (Bilski, 2012).

The localisation of the male sterility effector-fluorescent sperm marker fusion (Dmprot2-mCherry-FokI) in the male germline, was investigated by dissection of sexually mature OX4718 heterozygous males, reared off-tet and on-tet. OX4718B was not assessed; it was an impenetrant (fertile) line and was discarded before the study began. Heterozygous OX4718A male medfly were collected on eclosion (day 0) and kept in small cages (20 males per cage). They were re-fed on day three and dissected on day five. Testes from at least ten males per line were dissected by standard protocol in testis buffer, gently squashed under a coverslip and imaged at Cardiff University using phase contrast and fluorescence microscopy (Olympus BX50 microscope, Hamamatsu Orca 05G digital camera, and Olympus U-RFL-T fluorescence lamp). Spermatocytes, spermatids and sperm were imaged to identify cell-specific fluorescence, at 20x and 40x magnification. The fluorescent sperm marking system was partially functional, but not fit for purpose (not present in all mature sperm). The relatively poor performance of the sperm marker was not surprising, as a prior evaluation demonstrated that linker-free

fusions of Dmprot2-mCherry-FokI (OX4705) did not perform well in olive fly (Asadi, 2013). Effector localisation (Dmprot2-mCherry-FokI) was observed in spermatocytes, spermatids and sperm (**Figure 3.5**). Translation in spermatocytes indicated that Dmprot2-mCherry-FokI was present earlier than expected; the Dmprot2 5'UTR was anticipated to delay translation until spermatids were elongated (Jayaramaiah Raja and Renkawitz-Pohl, 2005). The effector was not completely repressed by tetracycline; and not uniformly expressed in all sperm. This was consistent with the observation that a small proportion of embryos hatched (1.1%). Although incomplete repression of the effector was observed (the mCherry fluorescence was detected even when the flies had been reared in the presence of tet), it did not appear sufficient to reduce fertility in tet-reared males, because the egg hatch rate was similar to WT. This was probably because FokI requires dimerisation for activity, and hence a direct correlation of concentration and activity is not expected. In terms of fluorescent sperm marking, expression in spermatocytes was not problematic per se. However, failure to detect fluorescence in an overwhelming majority of sperm were factors that needed to be solved by redesign of the sperm marking system. Finally, fluorescent marker expression in spermatocytes was nuclear, but in spermatids the whole cell was labeled, not just the nucleus. This suggests that the fusion protein is not behaving as expected.

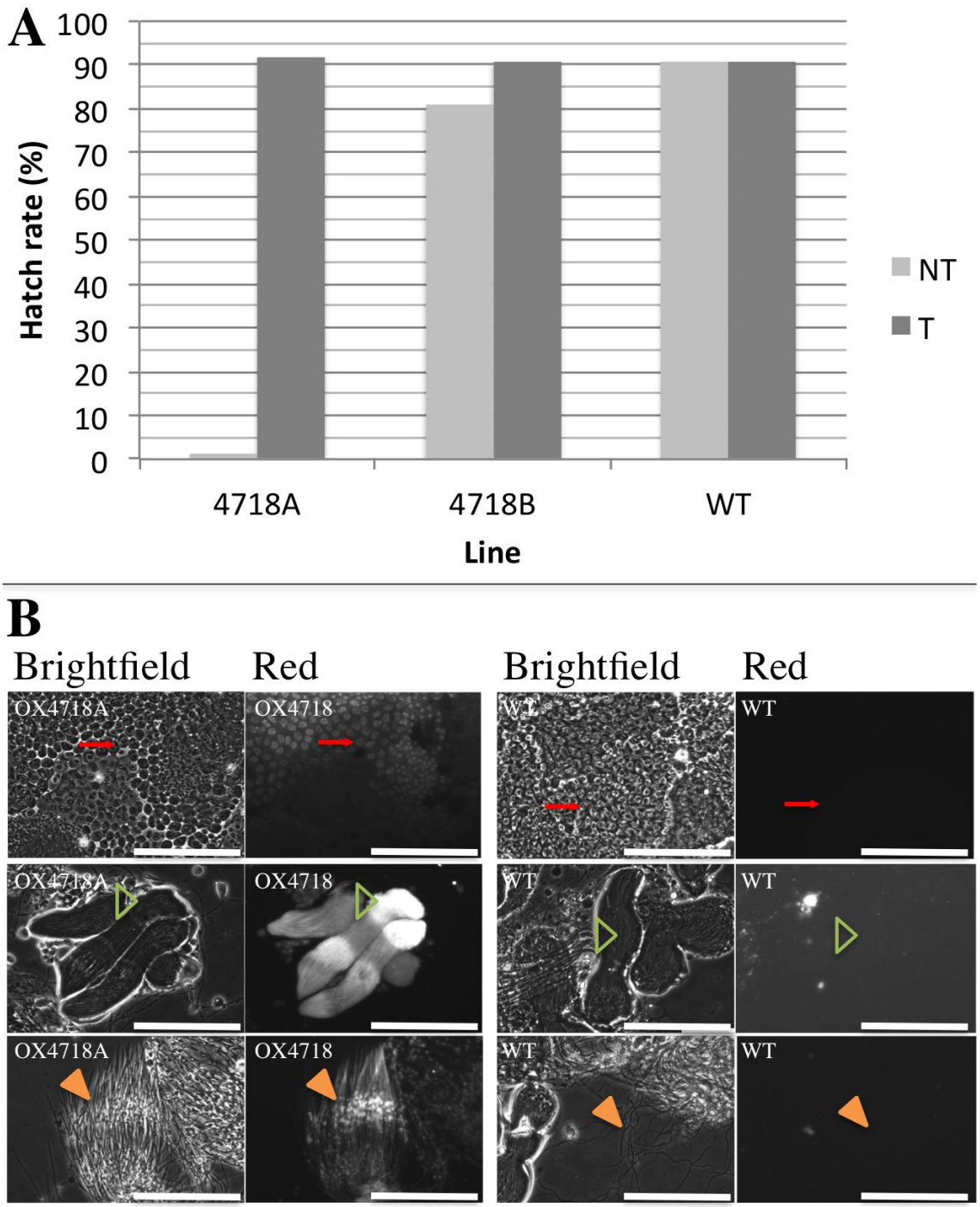


Figure 3.5. OX4718 male sterility and sperm marking phenotypes in heterozygous males (continued on next page). **(A)** Egg hatch rate assay indicates a penetrant and repressible male sterile phenotype in OX4718A but not OX4718B (Bilski, 2012). **(B-D)** Fluorescence and phase contrast microscopy of OX4718A and WT testes [40x magnification]. **(B)** Spermatocytes (arrow), spermatids (unshaded arrowhead) and mature sperm (shaded arrowhead) are visibly marked in OX4718A. Scale bars: 100 µm.

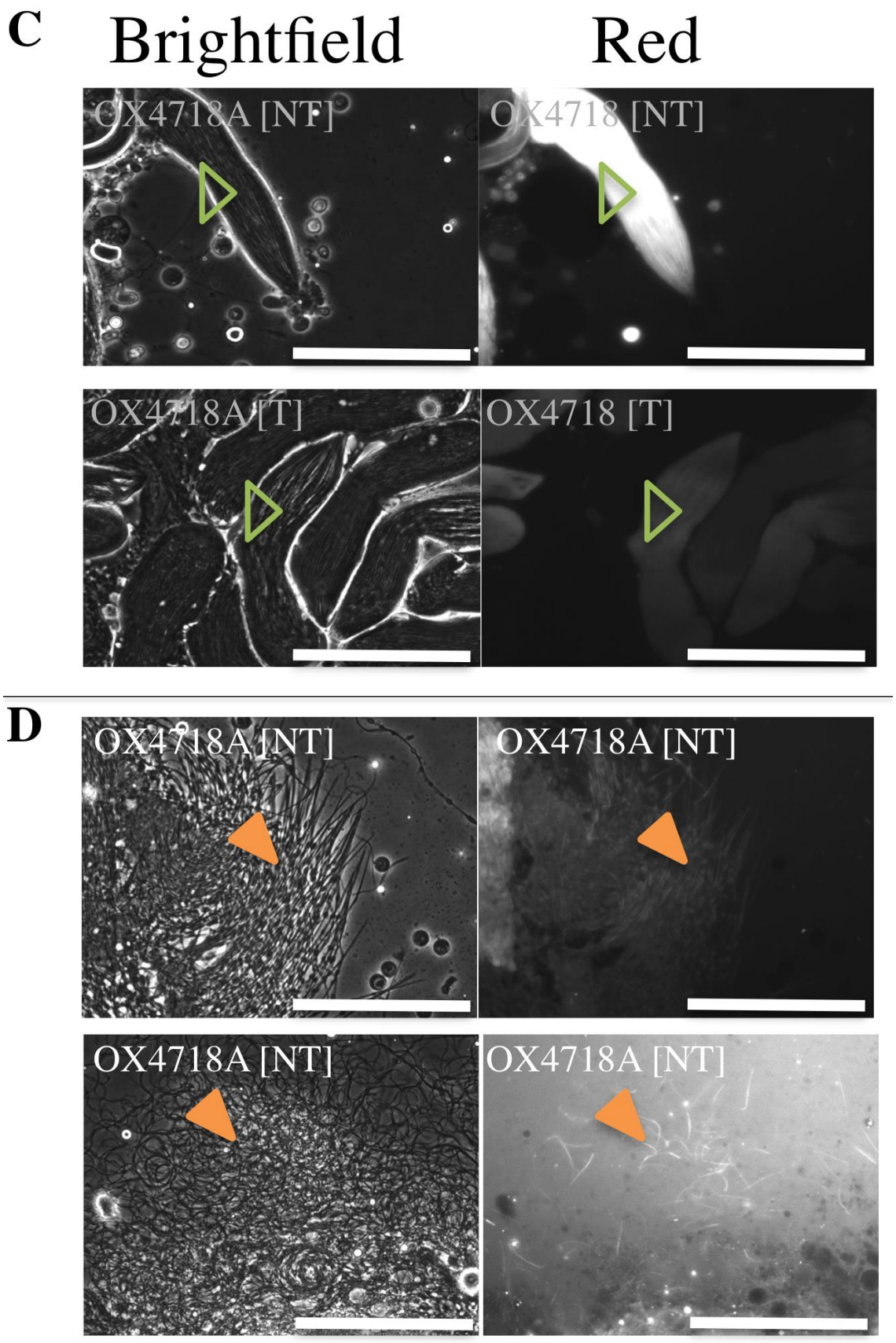


Figure 3.5. OX4718 male sterility and sperm marking phenotypes in heterozygous males. (**C**) Marking in spermatids (unshaded arrowhead) is visible both off-tet (NT) and on-tet (T), indicating that tetracycline does not fully repress effector expression. (**D**) Expression of the effector is not consistent; most sperm (shaded arrowhead) are not marked. Scale bars: 100 μm.

3.4 Immobilisation of the OX4718A insertion and re-assessment of the repressible male sterility phenotype

The penetrant and repressible male sterility phenotype, and fluorescent body and sperm markers are key biosafety features. Penetrant sterility prevents vertical transmission of the engineered genetic elements into the wild population; and the markers facilitate traceability of the engineered males in the wild (ie. they can be differentiated from wild males). Theoretically, the expression profile of these features could be removed, attenuated or otherwise altered by re-transposition to a novel insertion site. Furthermore, it is theoretically possible that the presence of transposable ends could facilitate horizontal gene transfer, for instance to an invading retrovirus and thereafter to novel insect species (Lohe et al., 1995), (Silva et al., 2004). Therefore, a unique selling point of this repressible male sterility platform is the ability to immobilise the transgenic insertion, by removal of sequences required for transposition from the *piggyBac* vector (Dafa'alla et al., 2006). This is anticipated to hasten the regulatory process and improve public acceptance of the product.

OX4718 is a composite transposon with two pairs of *piggyBac* ends that self-excise ("resolve"), when crossed to a transgenic line expressing *piggyBac* transposase in the germline (OX3133: Cchsp70-pB transposase-pB 3'UTR). Fluorescent markers at each *piggyBac* end pair (left: HR5IE1 promoter-**AmCyan**-BmHsp83 3'UTR; right: HR5IE1 promoter-**zsGreen**-DmK10 3'UTR) facilitate microscopic screening of *piggyBac* end excision (by marker loss). *piggyBac* end excision was performed largely as described previously (**Chapter 2**). All crosses were carried out in the presence of tetracycline. OX4718A heterozygous males were crossed to OX3133 heterozygous females. F_1 progeny carrying both constructs (OX4718A/OX3133) were selected by fluorescence microscopy (DsRed2 & PhiYellow expression). OX4718A/OX3133 males were then crossed to WT females; the reciprocal cross was also performed as excision can occur in both the male and female germlines. F_2 progeny were screened by fluorescence microscopy for the absence of zsGreen and AmCyan.

Thereafter, genomic DNA was extracted from four apparent positives to confirm end loss by PCR; all individuals had indeed lost both ends (**Figure 3.6**). A single transgenic OX4718A-resolved individual was backcrossed to WT for two generations to re-establish a genetically diverse transposon-free transgenic line. The male sterility phenotype was re-assessed by egg hatch rate assay at this stage, because it was possible that the transposon had remobilised to a novel genomic site. The assessment was essentially as previously described (**Chapter 2**). Fifteen heterozygous OX4718A-resolved males of each treatment (non-tet [NT] or tet [T] reared) were crossed to fifteen WT[NT] females. Controls were equivalent crosses with WT males and WT females. Crosses were performed in quadruplicate. A 24-hour egg collection (250 eggs) was performed four days later (four replicates per group, 1000 eggs total) and the hatch rate was assessed (day 8). The male sterility phenotype was equivalent to the unresolved OX4718A insertion (**Table 3.2**); it did not seem to have been affected by *piggyBac* end excision. Interestingly, three of four OX4718A-resolved [NT] cages did not give rise to hatching progeny; in one cage 6% of eggs hatched. Assuming there was no contamination with tetracycline, this may indicate that spermatocytes occasionally do not accumulate sufficient effector transcript, and the descendent sperm remain viable. No significant difference in male fertility was observed between crosses involving OX4718[T] and WT[T] males, indicating that the sterility is fully repressible by the addition of tet. However, statistically significant variation in hatch rate was observed in every group, when performing within-group testing on individual replicates (**Table 3.3**). This indicated that improving the reproducibility of the assay would improve the validity of future analysis. An optimisation assay later confirmed that survival was improved, when less water was applied to the moist filter paper in the hatching chamber. When this was adopted in subsequent experiments (Chapter 5), it was rare that significant variations across replicates of the same group were observed.

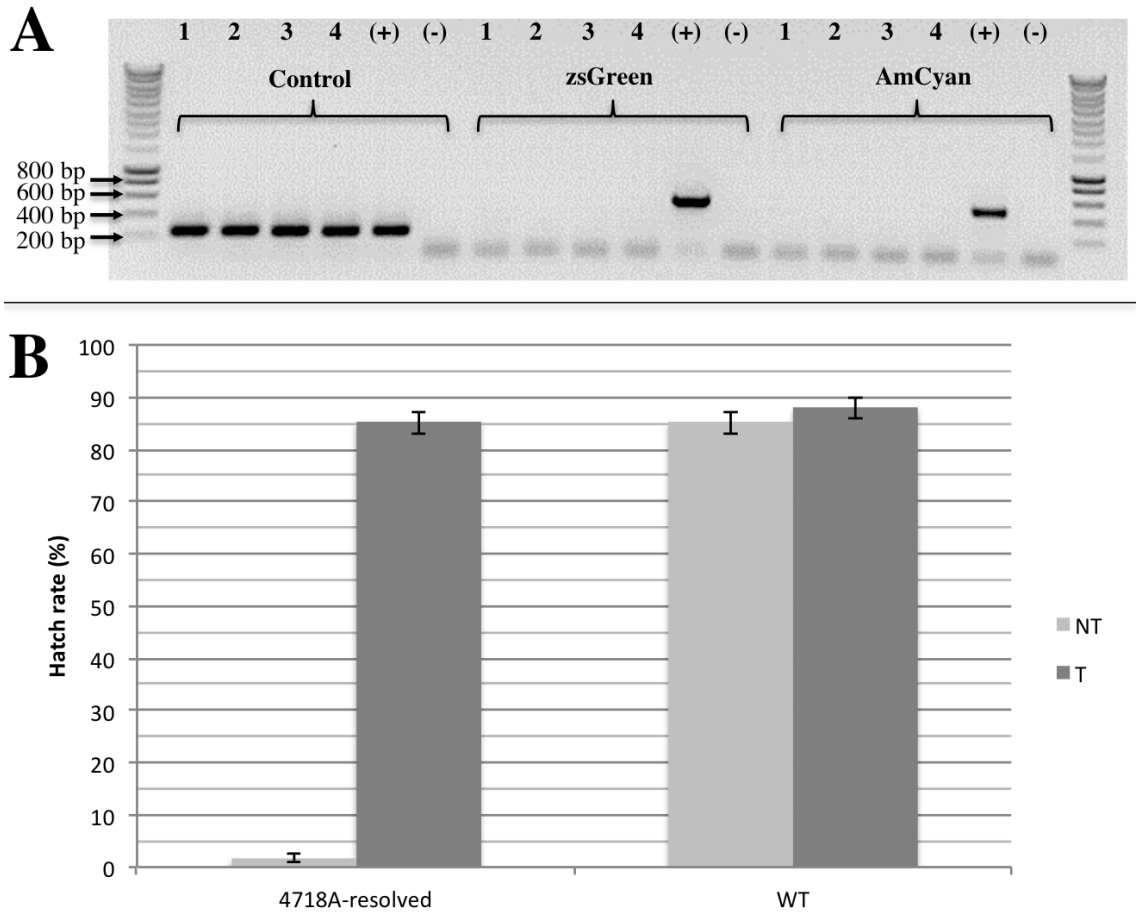


Figure 3.6. Immobilisation of the OX4718A insertion by removal of piggyBac ends ("resolution") and re-assessment of the repressible male sterile phenotype. Four OX4718 individuals apparently lacking piggyBac ends were assessed by PCR for loss of the zsGreen end and AmCyan end with PCR. Sequence within the central element was amplified as a control. Expected sizes: central (251 bp), green (551 bp), cyan (485 bp). Controls were water (negative, -) and OX4718 unresolved pupal gDNA (positive, +). All individuals lacked ends. One individual was backcrossed to WT for two generations to re-establish a transposon-free transgenic line (OX4718A-resolved). (**B**) Re-assessment of the repressible male sterile phenotype of OX4718A after immobilisation (OX4718A-resolved) demonstrates complete repressibility and a similar penetrance to the previously characterised line. Error bars: 95% CI (Wilson).

Table 3.2 Penetrance and repressibility of the male sterility phenotype in OX4718A-resolved

Line	**Mean hatch rate (± SE)**		**Penetrance**			**Repressibility**		
	NT	**T**	%	X^2	$P_{[df]}$	%	X^2	$P_{[df]}$
OX4718A-resolved	1.6 ± 0.4	85.3 ± 1.12	98	1423	**< 0.001**$_{[1]}$	100	3.4	0.07$_{[1]}$
WT	85.2 ± 1.12	88.1 ± 1.02						

Penetrance and repressibility were calculated relative to their respective control hatch rate, WT [NT] or [T]. Significance values were from chi-square tests of the numbers of hatched and unhatched eggs, relative to the respective values for the control (WT [NT] or [T]). SE: standard error. Df: degrees of freedom.

Table 3.3 Biological variation in the OX4718-A assay

Line	**NT**						**T**					
	Hatch rate (%)				**Significance**		**Hatch rate (%)**				**Significance**	
	R1	R2	R3	R4	X^2	$P_{[df]}$	R1	R2	R3	R4	X^2	$P_{[df]}$
OX4718A-resolved	0	0	0	**6.4**	36.2	**< 0.001**$_{[3]}$	**74.4**	82.4	89.6	94.8	47.0	**< 0.001**$_{[3]}$
WT	**65.6**	90.4	91.6	93.2	102.3	**< 0.001**$_{[3]}$	**80.4**	87.6	88.8	95.6	27.7	**< 0.001**$_{[3]}$

Replicate is abbreviated as "R". Outliers in boldface. Significant variation was indicated by chi-square testing the number of hatched and unhatched eggs, between the four replicate values for each group.

3.5 Establishing a homozygous OX4718A-resolved line

Homozygosity of the transgenic insertion is required for product lines, because it removes the need to screen transgenics during mass-rearing and may enhance the penetrance of male sterility by doubling copy number of the effector. A homozygous colony is easily established by PCR to genotype founders, but typically requires the sequences flanking the insertion, to differentiate heterozygotes and homozygotes. The OX4718A-resolved genomic flanking sequence (**Figure 3.7**) was isolated by Caroline Phillips (Oxitec Molecular Team) by restriction digest, ligation of an adapter of known sequence; and sequencing across the adapter, flanking genomic DNA, and transgenic expression construct. Unfortunately, a parallel analysis (next section) revealed that sperm transfer in OX4718A was deficient, limiting its usefulness as a product line. We therefore aborted homozygosis.

OX4718A-res (5′ flanking)
ggttcaaactttacatgtgagcaatcagtgtttcagtaaaagttttattgaatgaaatgataatataa
tgaattaaaattaataggaattaataaaaaaattctcaaatatccaaatcatgtttaaggtggtttta
cttggaaattttccacaccaaactttgacagtaaacaccctagaatcgtttaatcttgccactggcat
acaaggtattgagcgtgcagctttt**TTAA**

OX4718A-res (3′ flanking)
TTAAattactcttggggctatcttctaagcgagagctataaatacatagtgcaacatttttcgggtca
tcgaagccaaaaagattttcgagatcagcagttgacagtgcgaggggctgttggtggttagagagggt
ccatcatctc

Figure 3.7. Flanking sequence upstream (5') and downstream (3') of the OX4718A-resolved insertion with terminal TTAA sites indicated.

3.6 OX4718A-resolved heterozygous males compete well against WT males for mates, but transfer sperm with reduced motility, at a lower quantity

Fluorescence microscopy had confirmed translation of the male sterility effector-fluorescent sperm marker in spermatocytes (Dmprot2-mCherry-FokI), which indicated that undesirable effects on the quantity or motility of sperm transferred to females were possible. This could potentially encourage mated females to seek re-mating with other males (if the extent of sperm transfer was not adequate). Therefore, we next assessed the mating competitiveness of OX4718A-resolved males and the health of sperm transferred upon mating. OX4718A-resolved heterozygous males, WT males and WT females (all reared off-tet) were collected on eclosion after quality controlling the rears by mean pupal mass (> 7 mg), according to IAEA guidelines (FAO et al., 2003). On day 5, fifty individuals of each group were released into a Bugdorm cage (five replicates) and the duration of mating for each pair recorded. The male of each pair was genotyped (presence or absence of the AlMAct-dsRed2 fluorescent marker), to assess RSI (the proportion of mating events initiated by transgenic males). An RSI of 0.5 would indicate that OX4718A-resolved and wildtype males are equally competitive. To assess sperm transfer, females mated for > 60 minutes in the experiment were randomly selected and dissected at Cardiff University 24-48 hours later (same microscope as previously described, at 10x and 20x magnification).

The five replicates of the mating competition assay were pooled after verifying that there were no significant differences between the replicates, in terms of female mating preference (chi square test, $\chi^2 = 1.5352$, df = 4, p = 0.82). Females did not preferentially mate with either WT or OX4718A-resolved males (RSI = 0.5, $\chi^2 = 0.005$, df = 1, p = 0.95; **Table 3.4**). OX4718 males mated for 8 minutes longer on average (114 min) relative to WT males (106 min). This small effect was statistically significant (independent samples, two-tailed t-test; t = -2.0597, df = 195, p = 0.04). However, the effect was no longer significant when t-testing was re-performed on individual replicates. This indicates an artefactual difference, probably caused by handling differences in the three people (Ryan Turkel, Charilaos Megas and Ben Granville) working together to conduct the study. The mean durations of mating for both genotypes were less than the average for mass-reared Medfly males reported elsewhere, approximately 135 minutes (Shelly and Kennelly, 2002). In subsequent analyses (**Section 4.3.3**), the reported duration of mating (138-142 minutes) was greater than this published value (135 minutes). This suggests that experience with the assay (perhaps

the ability to delicately handle pairs in copula), had a substantial effect on the duration of mating.

Table 3.4: Mating preference of WT females in OX4718A-res competition assay

Replicate	**Mating preference**					**X^2**	**$P_{[df]}$**
	Transgenic	**WT**	**Total**	**RSI**	**Unmated**		
1	20	25	45	0.44	5	0.56	$0.46_{[1]}$
2	22	25	47	0.47	3	0.19	$0.66_{[1]}$
3	21	23	44	0.48	6	0.09	$0.76_{[1]}$
4	25	15	40	0.63	10	2.5	$0.11_{[1]}$
5	21	20	41	0.51	9	0.02	$0.88_{[1]}$
Total	**109**	**108**	**217**	**0.50**	**33**	**0.005**	**$0.95_{[1]}$**

13/15 females crossed to WT males had detectable sperm; in all but one individual they were too numerous to count. In contrast, 12/16 (75%) of females crossed to OX4718A-resolved males lacked detectable sperm. In the four instances where OX4718A-resolved sperm were observed, fewer than 100 were present in the entire female genital tract. Hundreds or thousands are typically present, when the male to which females are mated, is WT (Taylor and Yuval, 1999), (Twig and Yuval, 2005). OX4718A-resolved sperm frequently had unusual morphology (uncurled and presumably dead), were generally immotile, and never detectably marked (**Figure 3.8**). Occasionally, weakly swimming sperm were found in the oviduct. These appeared to be trapped and unable to pass into the spermathecae. Therefore, it appears that expression of the male sterility effector (Dmprot2-mCherry-FokI) in OX4718A-resolved males promotes genomic inviability of sperm as intended. However, in most instances, it appears to reduce the quantity and motility of sperm transferred to the female reproductive tract.

Therefore, it is likely that a large proportion of embryos that do not develop in OX4718A-resolved hatch assays are unfertilised, rather than inviable as a result of paternal effect lethality. It is not entirely clear why certain sperm are less affected than others; but it seems likely that expression levels of the effector in sperm are not perfectly uniform. This was corroborated by the localisation pattern of the Dmprot2-mCherry-FokI effector, which marked particular sperm and spermatids more brightly than others. Curiously, marking was not observed in mature sperm that were transferred to WT females from OX4718A-resolved males; though it was occasionally visible in testes (**Figure 3.5**). A speculative explanation is that the nuclease activity of Dmprot2-mCherry-FokI fragments the sperm genomic DNA, spatially de-aggregating the fluorescent proteins and therefore reducing their visibility. The presence of weak but detectable fluorescence in the male germline is probably related to two factors: (1) a greater concentration of spermatids and sperm (ie. cells in which the fluorescence should be visible); and (2) the presence of newly forming sperm, which may have been exposed to the Dmprot2-mCherry-FokI nuclease for a lesser time period (it is possible that the genome is relatively intact).

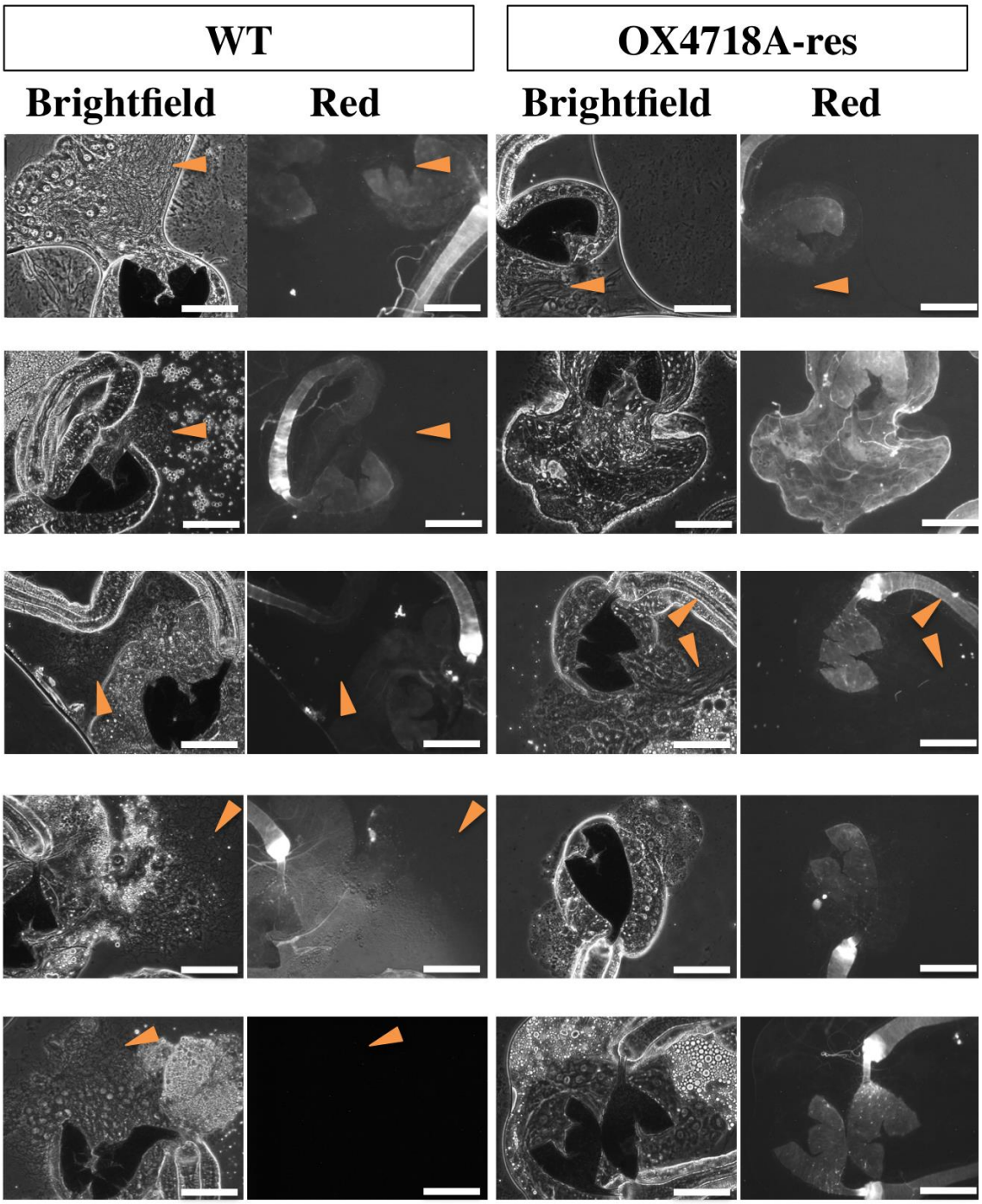


Figure 3.8: OX4718A-resolved heterozygous males transfer unmarked sperm in low quantity. Fluorescence and phase contrast microscopy of spermathecae from WT females mated to WT or OX4718A-resolved males. All individuals were confirmed to have mated for at least one hour. Representative images from ten individuals shown (five per genotype). OX4718A males transferred fewer sperm (shaded arrowhead), on average (none detected in 12 of 16 instances; 2 in 15 instances for WT). Sperm were not detectably marked. Therefore, the sperm marking system needed a complete redesign and the male sterility effector would be potentially improved by altering the Dmprot2-FokI effector for delayed translation. Scale bars: 100 μm.

Interestingly, the observation of a low, but not zero, hatch rate in crosses of WT females to OX4718 males reared-off tet, indicates that a minority of sperm retain a genome sufficiently intact to mediate hatching of the fertilised embryo. Such an effect could be mediated by stochastic variation in expression of the tet system across cells, or by the spatial dynamics of distribution of effector transcript and protein, as spermatocytes develop into sperm. The negative effect on sperm fitness was not unexpected, because the previous analysis demonstrated translation in spermatocytes (protamine-mCherry-FokI). As previously described, the 5'UTR is a key mediator of translational repression in *Drosophila melanogaster* protamine-like genes (Barckmann et al., 2013). The OX4718 construct retains a portion of Dmhsp70 5'UTR (89 bp associated with the Dmhsp70 minipromoter (tetO21-Dmhsp70 minipromoter[+89 bp 5'UTR]-Dmprot2 5'UTR). As this gene is translated earlier than Dmprot2 (embryonically), it was possible that the Dmhsp70 5'UTR fragment may have attenuated the ability of the adjacent Dmprot2 5'UTR, to mediate appropriate translational delay. It was additionally possible that the Dmprot2 5'UTR was not functional in Medfly. Clearly, a redesign of this system would be preferable, to facilitate a male mating phenotype similar to WT.

3.7 Establishment and Mendelian assessment of transgenic OX5036 lines

OX5036 (tetO21-Dmprot2 ORF[no introns]-SG4 linker-FokI; Ccβ2tubulin[short]-Cchsp83-tTAV-T2A-nls-zsGreen-nls) was primarily evaluated to test the feasibility of a new sperm marking design, as a penetrant and repressible male sterility effector had been previously described (OX4353/4718). The alterations made to the male sterility effector (Dmprot2 ORF[no introns]-SG4 linker-FokI), relative to the previous design (OX4718) were: the removal of introns from Dmprot2, alterations to the C-terminal end of Dmprot2, and the addition of an SG4 linker between Dmprot2 and FokI (outlined fully in **Section 3.2**). These were considered to possibly enhance the penetrance of sterility; a complete lack of embryonic hatching would have been preferable. The tetracycline-repressible transcriptional regulator was altered to include a linked fluorescent sperm marking system **(**tetO21-Ccβ2tubulin promoter[short]-Cchsp83-tTAV-**T2A-nls-zsGreen-nls**).

Microinjection and backcrossing to WT (**Tables 3.5-3.6**) were performed as described in **Chapter 2**. Buffered injection mixes were prepared with *piggyBac* helper transposase plasmid OX3022 (300 ng/µl) and OX5036 (600 ng/µl). Successful microinjection was confirmed by transient expression of the AlMAct-dsRed2

transformation marker. The adult survival rate (46%) was higher than the average rate for Medfly microinjections at Oxitec (25%) (Gregory et al., 2016). Fourteen lines were generated. Ten were assessed for penetrance of the male sterility phenotype (egg hatch assay). The three most penetrant lines and an impenetrant line were assessed for fluorescent sperm marking in the male germline (fluorescence microscopy). Mendelian properties of each insertion were assessed by marker inheritance in G_2 progeny, as described previously (**Table 3.7**). A single insertion provides a 1:1 ratio of transgenic to WT progeny. Crosses of a male transgenic individual with a single insertion will yield male-only transgenic progeny if Y-linked, and female-only transgenic progeny if X-linked.

Table 3.5: Microinjection logistics for OX5036

Embryos	Larvae	Pupae	Adults	Lines
1186	545 (46%)	433 (37%)	391 (33%)	14 (3.6%)

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (transgenic lines/G0 adults crossed).

Table 3.6: G_0 backcrosses to establish OX5036 lines

OX5036A	**19 ♀ x 19 WT ♂**	OX5036B	10 ♂ x 30 WT ♀	OX5036C	10 ♂ x 30 WT ♀
OX5036D	11 ♂ x 30 WT ♀	OX5036E	10 ♀ x 10 WT ♂	**OX5036F**	**10 ♀ x 10 WT ♂**
OX5036G	10 ♂ x 30 WT ♀	OX5036H	10 ♀ x 10 WT ♂	OX5036I	10 ♂ x 30 WT ♀
OX5036J	10 ♂ x 30 WT ♀	**OX5036K**	**10 ♀ x 10 WT ♂**	OX5036L	10 ♂ x 30 WT ♀
OX5036M	**10 ♂ x 30 WT ♀**	OX5036N	10 ♀ x 10 WT ♂	**OX5036O**	**6 ♀ x 6 WT ♂**
OX5036P	10 ♀ x 10 WT ♂	OX5036Q	7 ♂ x 30 WT ♀	OX5036R	10 ♂ x 30 WT ♀
OX5036S	**11 ♂ x 30 WT ♀**	OX5036T	10 ♂ x 30 WT ♀	**OX5036U**	**20 ♀ x 20 WT ♂**
OX5036V	**20 ♀ x 20 WT ♂**	OX5036W	7 ♀ x 7 WT ♂	OX5036X	10 ♂ x 30 WT ♀
OX5036Y	**20 ♀ x 10 WT ♂**	OX5036Z	10 ♂ x 30 WT ♀	OX5036AA	4 ♂ x 12 WT ♀
OX5036AB	4 ♀ x 4 WT ♂	**OX5036AC**	**20 ♀ x 10 WT ♂**	**OX5036AD**	**20 ♀ x 10 WT ♂**
OX5036AE	**12 ♀ x 10 WT ♂**	**OX5036AF**	**11 ♂ x 30 WT ♀**	**OX5036AG**	**13 ♂ x 30 WT ♀**
OX5036AH	10 ♂ x 30 WT ♀	OX5036AI	11 ♂ x 30 WT ♀		

Underlined pools yielded transgenics. Transgenic lines were established from a single individual and assessed from OX5036A, K, M, O, S, V,Y, AC, AD & AG.

Table 3.7: Mendelian analysis of OX5036 transgenic lines

Line	**Sex**	**Copy number**		**Sex-linkage**		**Summary**		**Penetrance**
		Transgenic (%)	n	Sex ratio (M/F)	n	Copies	Location	
OX5036V	♂	53.2	139	1.1	65	1	Autosome	95%
OX5036S	♂	58.8	136	0.7	75	1	Autosome	92%
OX5036O	♂	48.9	399	0.8	168	1	Autosome	91%
OX5036A	♂	57.5	120	1.0	120	1	Autosome	89%
OX5036K	♂	51.4	109	1.4	40	1	Autosome	88%
OX5036M	♂	48.1	79	1.3	35	1	Autosome	86%
OX5036Y	♂	51.5	299	1.0	131	1	Autosome	77%
OX5036AD	♂	50.0	34	0.7	15	1	Autosome	29%
OX5036AC	♂	50.2	243	**Female only**	118	1	**X**	0%
OX5036AG	♂	60.3	179	**Male only**	105	1	**Y**	0%

All lines were single insertions. OX5036AC was X-linked and OX5036AG was Y-linked. All other insertions appeared to be autosomal. Penetrance and repressibility of the male sterility phenotype were assessed in the subsequent section. “n”: number of individuals assessed.

3.8 OX5036 heterozygous lines demonstrate reduced penetrance of male sterility, relative to prior constructs (OX4718 & OX4353)

Penetrance was investigated by egg hatch assay, essentially as previously described for OX4718 (**Section 3.3**). However, to identify highly penetrant lines for further study, the initial screen was performed with off-tet reared males only (we investigated the penetrance of sterility, but not repressibility). It was not subsequently necessary to investigate repressibility (by repeating the egg hatch assay with off-tet and on-tet reared males), because none of the lines demonstrated adequate penetrance. Twenty-five heterozygous OX5036 males reared off-tet [NT] were crossed to fifty WT[NT] females. The control was an equivalent cross with WT[NT] males and WT[NT] females. On day 4, three collections of 150-300 eggs (< 24 hours old) were taken from a 24 hour collection of the same cage (pseudo-replication). Eggs were left to hatch in a humid chamber as previously described. Hatch rate was scored on day 8.

Penetrance of the male sterility phenotype was highly insertion sensitive (**Table 3.8, Table 3.9, Figure 3.9**), varying widely across lines (0-95%). Interestingly, the only two completely impenetrant lines (OX5036AC & AG) were sex-linked. An insertion equally penetrant to OX4718A-resolved (98%) was not identified, despite screening 8 lines where the sterility effector was clearly expressed (reduction in egg hatch rate observed). OX5036V was the most penetrant line tested (95%). The extent of penetrance was deemed to be insufficient for commercial use, as the release of partially fertile transgenic males into the wild is contentious from a regulatory and practical perspective. It was found that two non-sterile transgenic lines (OX5036AC & AG) demonstrated significantly higher hatch rates than WT; there is no reasonable biological basis for this effect. For this reason, significance analysis of the differences in hatch rate between lines should be interpreted with caution (**Table 3.8**), even though the differences in hatch rates observed for replicates of the same line were not generally statistically significant (**Table 3.9**). Comparing the WT hatch rates (56%) observed in this study to those of other equivalent studies (70%-95%), suggested that the conditions in the hatching chambers were not ideal (Bilski, 2012), (Asadi, 2013). The results therefore indicated that penetrance was probably overestimated (because the hatching conditions used in the assay reduced the viability of eggs, relative to perfect conditions). Colleagues experienced in the assay suggested that the hatching chambers may have been maintained in an overmoistened state. In future experiments, we attempted to improve the method by more carefully checking the humidity of the chambers.

Table 3.8 Penetrance and repressibility of male sterility in OX5036

Line	**Mean hatch rate (± SE)**	**Penetrance**		
	NT	%	X^2	$P_{[df]}$
5036V	2.6 ± 0.6	95	573.1	**< 0.001**[1]
5036S	4.4 ± 0.7	92	509.6	**< 0.001**[1]
5036O	4.8 ± 0.8	91	485.1	**< 0.001**[1]
5036A	6.2 ± 0.8	89	485.5	**< 0.001**[1]
5036K	6.5 ± 0.9	89	460.6	**< 0.001**[1]
5036M	7.8 ± 1.0	86	416.0	**< 0.001**[1]
5036Y	12.7 ± 1.2	77	331.4	**< 0.001**[1]
5036AD	39.9 ± 1.9	29	38.5	**< 0.001**[1]
5036AC	62.7 ± 1.7	0	7.3	**0.006**[1]
5036AG	68.8 ± 1.6	0	27.2	**< 0.001**[1]
WT	56.1 ± 1.8			

Penetrance was calculated relative to the control hatch rate, WT [NT]. Significance values were from chi-square tests of the numbers of hatched and unhatched eggs, relative to the respective values for the control (WT[NT]). SE: standard error. Df: degrees of freedom.

Table 3.9 Biological variation in the OX5036 egg hatch assay

Line	**Hatch rate (%), NT**			**Significance**	
	R1	R2	R3	X^2	$P_{[df]}$
5036V	3.3	2.2	2.0	1.1	0.59[2]
5036S	5.0	4.4	3.9	0.37	0.83[2]
5036O	5.6	5.5	3.2	2.0	0.37[2]
5036A	8.2	5.8	4.8	3.1	0.22[2]
5036K	9.9	4.8	4.6	8.21	**0.02**[2]
5036M	10.2	7.3	6.1	3.15	0.21[2]
5036Y	13.5	13.1	11.6	0.51	0.78[2]
5036AD	45.1	41.0	35.9	3.7	0.16[2]
WT	58.5	57.4	52.0	2.4	0.30[2]
5036AC	65.4	62.0	60.4	1.6	0.46[2]
5036AG	69.6	69.0	67.9	0.2	0.90[2]

Replicate is abbreviated as “R”. Significant variation was assessed by chi-square testing the number of hatched and unhatched eggs, between the three replicate values for each group; it was only detected for OX5036K.

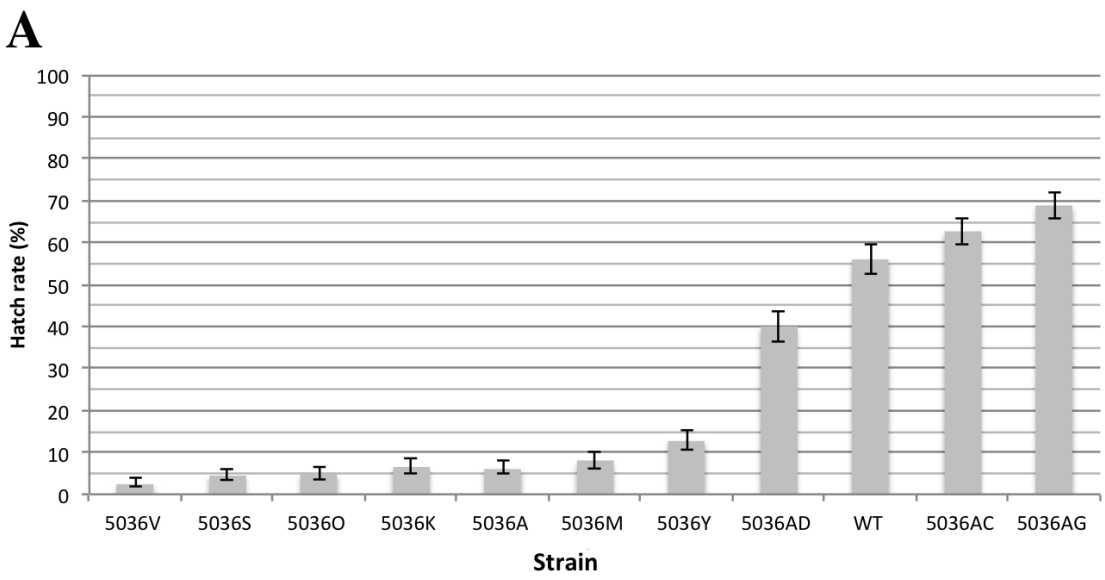


Figure 3.9. Assessment of the penetrance of the male sterility phenotype in OX5036 heterozygous males by egg hatch assay. Preliminary, off-tet only analysis of ten lines. Penetrance was highly insertion sensitive, varying widely across lines (0-95%). Error bars: 95% CI.

These results indicated that changes made to the Dmprot2-FokI effector were responsible for reducing penetrance. The removal of mCherry from the effector was not considered likely to be responsible, as a marker-free effector (OX4353) of similar construction routinely demonstrated highly or fully penetrant insertions. We therefore compared the architecture of the tetracycline-repressible transcriptional activators and the male sterility effectors of OX4353 and OX5036 (**Figure 3.10**). The presence of seven additional copies of tetO (tetO14 → tetO21) should not have altered the phenotype. If anything, it was predicted to make expression stronger because OX4353 was fully penetrant with fewer copies of tetO. The 3'UTR substitution (SV40 → Cchsp83) in the tetracycline-repressible transcriptional activator is very unlikely to have been responsible, because OX4718A demonstrated a fully penetrant and repressible phenotype with this 3'UTR. Additionally, it was unlikely that the removal of Dmprot2 introns from the open reading frame would reduce transcription, because intron-containing genes expressed in the male germline are frequently transcribed to a lesser extent (White-Cooper and Caporilli, 2013). Alterations made to the C-terminal region of the Dmprot2 coding sequence may have had an effect. In OX5036, the full wildtype sequence was used; in OX4353, the C-terminal lysine was removed and there were three residues (TMA) immediately preceding the fusion to FokI; these were absent in OX5036. However, we considered that the inclusion of SG4 linkers between Dmprot2 and FokI was the most likely factor, because their inclusion between mCherry-Dmprot2 and FokI (SG4 linker-mCherry-Dmprot2-SG4 linker-FokI) reduced the penetrance of male sterility in OX4801 (Asadi, 2013). Therefore, we eschewed the use of linker sequences in subsequent product design.

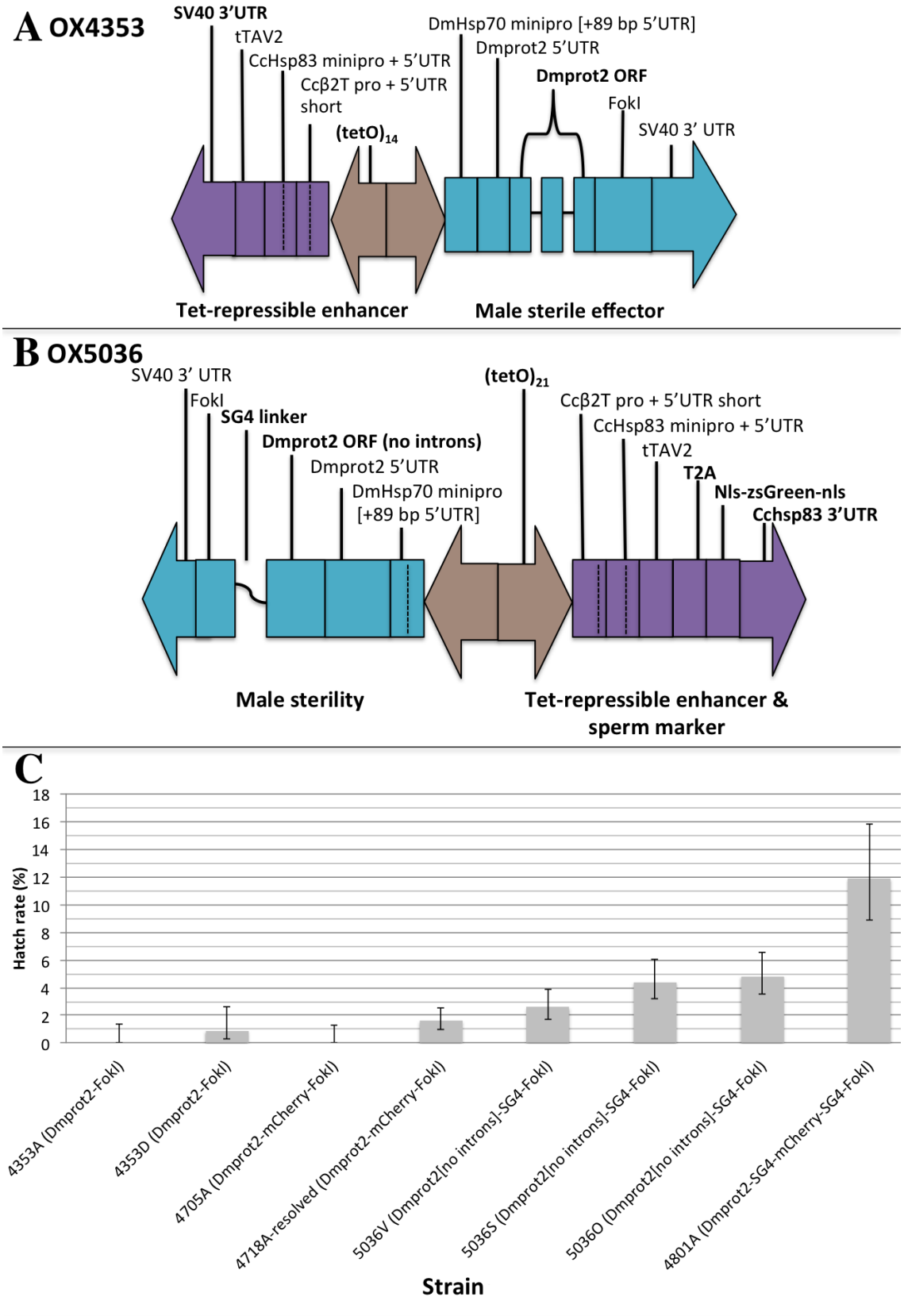


Figure 3.10. The inclusion of SG4 linkers in the OX5036 male sterile effector is the most likely cause of its systematic reduction in penetrance. (**A-B**) Comparison of male sterility effectors in OX4353 (**A**) and OX5036 (**B**). Dashes indicate promoter-5'UTR boundary. Relative to OX4353, OX5036 includes 7 extra copies of tetO; Dmprot2 introns were removed; the C-terminus of Dmprot2 was modified; and an SG4 linker was added. (**C**) Comparison of penetrance in strains with (OX5036 & OX4801) and without (OX4353, OX4705 & OX4718) SG4 linkers. OX4353, OX4718, OX4751 & OX5036 were evaluated in Medfly; OX4705, & OX4801 were evaluated in olive fly (error bars: 95% CI). Data for OX4353, OX4705, OX4751 & OX4801 is adapted from Asadi (2013).

3.9 The fluorescent sperm marking system in OX5036 is not functional

Despite demonstrating an unacceptably impenetrant male sterility phenotype, it remained possible that the OX5036 sperm marking system could be applied in future research and development (a reliable system had not been identified yet). We therefore assessed the three most penetrant lines (OX5036V, S & A) and an impenetrant line (OX5036AG) for fluorescent marker expression in the male germline, on- and off-tet. This analysis was essentially as described for OX4718A. Virgin OX5036 and WT males reared off-tet or on-tet were aged for 5 days. Dissection was performed on at least 10 males per line and imaged by fluorescence microscopy at Cardiff University. The 20x, rather than 40x, objective was used (to gather a larger number of spermatocytes, spermatids and sperm in the same image). Fluorescence intensity was inferior to OX4718A and reliably present in spermatocytes only (**Figure 3.11**). As expected, the brightness correlated with penetrance of male sterility (OX5036V, the most penetrant line, was brightest). Although the fluorescent sperm marking system was not suitable, analysis of reporter translation provided interesting mechanistic insights into the expression dynamics of its regulator (the tetracycline-repressible transcriptional activator: tetO21-Ccβ2tubulin [short]-Cchsp83-tTAV-T2A-nls-zsGreen-nls).

Fluorescent marking was visible in spermatocyte nuclei of penetrant lines (OX5036V, S & A). Spermatocytes were marked to a greater extent in the most penetrant line (OX5036V), suggesting that early accumulation of the transcriptional activator (tTAV) is associated with penetrant sterility, as expected. Furthermore, it suggests that the insertion sensitivity of the chimeric Ccβ2tubulin[short]-Cchsp83 fragment is an important factor in determining the penetrance of a given transgenic insertion. Not all spermatocytes were marked; this may explain why a minority of sperm escape the sterilising effect of the system. Marker expression in spermatids and sperm was only clearly visible in OX5036V, consistent with its highly penetrant sterile phenotype. Fluorescent marker expression was almost completely repressed by tetracycline in all lines, suggesting that basal germline expression of tTAV is low, as desired, at least in the male germline (preventing leakiness). It is not possible to directly infer the extent of expression of the male sterility effector (Dmprot2-FokI), as it was not associated with a fluorescent reporter. It would be interesting to investigate the relationship between tTAV and effector (Dmprot2-FokI) expression with a construct applying a secondary reporter (Dmprot2-mCherry). Because fluorescent sperm marking in OX5036 strains is weaker relative to strains expressing a protamine-marker fusion

(eg. OX4718), it appears that fusion to protamine or another DNA-binding protein is required for adequate fluorescent sperm marking.

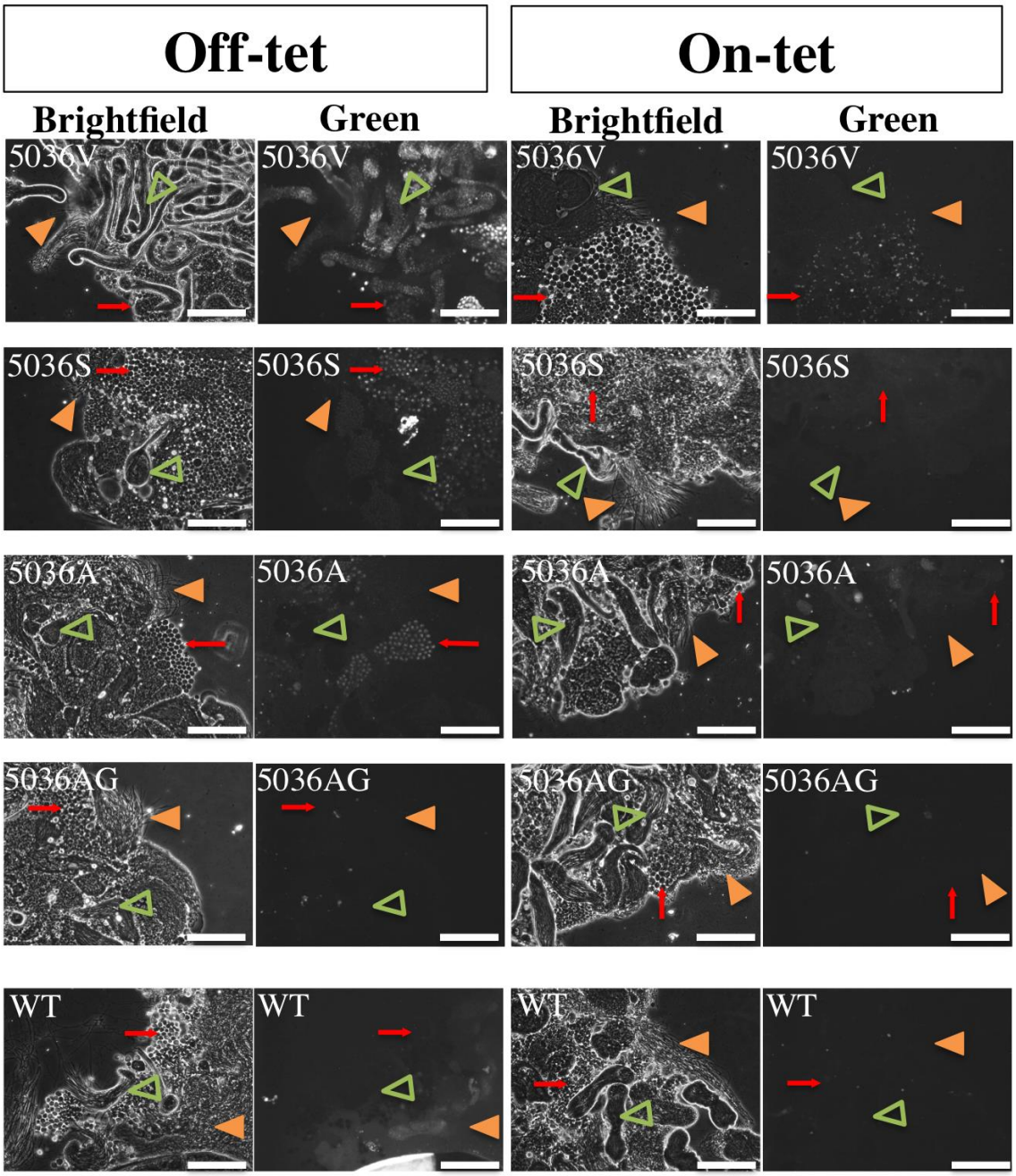


Figure 3.11. The OX5036 sperm marking system (tetO21-Ccβ2tubulin[short]-Cchsp83-tTAV-T2A-nls-zsGreen-nls) demonstrates appropriate localisation and expression of the transcriptional activator (tTAV), but is not suitable for use in sperm marking. Lines arranged in decreasing order of sterile penetrance (OX5036V: 95%; S: 92%; A: 89%; AG: 0%). Fluorescent reporter localisation is visible in spermatocyte nuclei of penetrant lines (OX5036V, S & A) reared off-tet; fluorescence is almost completely repressed when reared on-tet. Annotations: spermatocytes (arrow); spermatids (unshaded arrowhead); sperm (shaded arrowhead). Scale bars: 100 µm.

3.10 Conclusions

The primary objective of this study was to identify expression systems to suitably express effectors for fluorescent sperm marking and repressible male sterility. It was desirable to engineer systems that would minimally affect male reproductive competitiveness, in all aspects except the ability to support embryonic development. This was an important consideration, because it had been demonstrated that sterile males applied in prior mass release programmes were adversely affected by irradiation, which limited their ability to mate females in the field (Shelly et al., 1994), (Alphey, 2006). Specifically, it was highly preferable that sperm expressing protamine-FokI would retain the ability to be normally transferred to the spermathecae and fertilisation chamber, and thereafter enter the embryo. This would be expected to provide the cues associated with a typical fertile mating, lowering the probability of further re-mating with fertile, wild males (Mossinson and Yuval, 2003).

To this end, we evaluated two constructs (OX4718 & OX5036). In OX4718, the repressible male sterility effector was fused to mCherry, as a single molecule system for sterility and fluorescent sperm marking (tetO21-Dmhsp70 minipromoter-Dmprot2-mCherry-FokI). A variant of this effector that did not incorporate an mCherry marker (OX4353: tetO21-Dmhsp70 minipromoter-Dmprot2-FokI) had previously mediated penetrant and repressible male sterility in Medfly (Asadi, 2013). The sterility effector in OX5036 was, similarly, not associated with mCherry. Further changes were made to the male sterility system of OX5036 (tetO21-Dmhsp70 minipromoter-Dmprot2[no introns]-SG4-FokI), in an attempt to improve penetrance of male sterility. Introns are associated with reduced expression in some male germline genes (White-Cooper and Caporilli, 2013). Therefore, their removal from Dmprot2 could potentially enhance expression. The C-terminal end of Dmprot2 was also altered slightly relative to prior constructs and an SG4 linker sequence was added (Dmprot2-SG4-FokI), as previously described. Finally, the tetracycline-repressible enhancer regulating the effector in OX5036 was altered to include a linked fluorescent sperm marker (tTAV-**T2A-nls-zsGreen-nls**). tTAV and nls-zsGreen-nls were translated from the same mRNA as two polypeptides, and separated at the T2A sequence.

Sterility was penetrant and fully repressible in OX4718A, although the fluorescent sperm marker performed poorly (it did not localise to the majority of sperm nuclei). OX4718A-resolved males demonstrated a highly competitive phenotype in lab-scale mating competition assays; WT females demonstrated no significant preference

for WT males or OX4718A-resolved males. It should be noted, however, that the mating behaviour of Medfly reared in the lab is not an accurate indication of the wild equivalent (Liimatainen et al., 1997), (Briceño and Eberhard, 1998). Furthermore, the conditions of the assay are highly artificial. For instance, flies are assayed at a high population density, which is associated with a greater rate of re-mating (Shelly et al., 2004). Furthermore, the use of wild-type males as competitors (rather than wild-caught males) will tend to overestimate the competitiveness of the sterile line, because the wild-type strain is more likely to demonstrate lab adapted behaviour and the equivalent reductions in fitness associated with lab colonisation and rearing (Liimatainen et al., 1997). In greenhouse trials that simulate field conditions more accurately, it has been shown that strains which previously appeared to have no defects in competitiveness (as indicated in lab-scale assays), are substantially less able to compete for mates than their wild counterparts (Martha Koukidou, personal correspondence). Although it is known that the genetic changes associated with lab adaptation can reduce mating competitiveness or alter mating behaviour (Blay and Yuval, 1997), (Alphey, 2006); sterilisation by irradiation is directly associated with reductions in male competitiveness and sperm transfer, which we attempted to avoid by selective expression of protamine-FokI in the male germline.

Despite this attempt, dissection of WT females mated to OX4718A-resolved males demonstrated that expression of Dmprot2-mCherry-FokI was associated with a reduction in the number of sperm transferred, in most instances. The sperm present frequently demonstrated an unusual, uncurled morphology and were often immotile, indicating that the sperm were weakened or dead. We also noticed that sperm were prone to becoming trapped in the oviduct, consistent with a reduction in fitness. This failure to behave normally in the female reproductive tract may indicate that the primary factor mediating sterility in the system is not a result of paternal effect lethality, but rather a lack of sperm transfer, and of fertilisation itself. An investigation of effector localisation (tetO21-Dmprot2-mCherry-FokI) by fluorescence microscopy provided limited explanation of this effect. Reporter expression was observed in spermatids and sperm, as intended, but also spermatocytes. The theoretically perfect system would demonstrate substantially later translation (ie. not in spermatocytes), to minimise the possibility of negative effects on sperm production, motility and morphology. OX4718 contains a partial Dmhsp70 5’UTR, immediately preceding the Dmprot2 5’UTR

(tetO21-Dmhsp70 minipromoter[+89 bp 5'UTR]-Dmprot2 5'UTR). This may act to enhance early translation (as observed when the Ccβ2tubulin 5'UTR was truncated and stitched to Cchsp83 minipromoter-5'UTR in OX4282), or prevent a translational delay mediated by Dmprot2 5'UTR. It was additionally possible that the Dmprot2 5'UTR is not sufficient to mediate an equivalent translational delay in the male germline of Medfly, as it does in *D. melanogaster* (Jayaramaiah Raja and Renkawitz-Pohl, 2005).

Variations in the intensity of fluorescent marker localisation (tetO21-Dmprot2-**mCherry**-FokI) in sperm of OX4718A-resolved indicated that expression levels of the effector in sperm are not perfectly uniform. This was corroborated by the observation that only a small minority of sperm are motile, and that a small number of sperm are able retain a genome sufficiently intact to allow hatching of the fertilised embryo (1% hatch rate). The observation that sperm transfer to some females was essentially zero indicated that in many instances, the dominant mode of sterility was not paternal effect lethality as anticipated, but rather a lack of fertilisation. This appeared to be mediated in some instances by a severe deficiency in sperm transfer, and in other instances by poor motility of the transferred sperm. This is significant, because there is an inverse relationship between the number of sperm transferred and the tendency to re-mate in Medfly (Mossinson and Yuval, 2003). Therefore, transfer of sperm is essential; but transfer of weakened sperm in adequate quantities might be expected to induce female refractoriness to re-mating, at least partially. It would be interesting to further investigate the proportion of embryos that demonstrate a paternal effect lethal phenotype, for instance by visualizing embryonic development with nuclear staining. An assay based on staining was later developed and successfully applied to investigate the effect of protamine-FokI expression on embryos fertilised with sperm of transgenic lines (**Section 5.2.6**).

In contrast to OX4718A, the OX5036 male sterility effector was associated with a systematic reduction in the penetrance of sterility (no lines were more than 96% penetrant). A highly similar construct was previously shown to mediate highly penetrant sterility (OX4353; two of four lines were > 99% penetrant). Comparison of the OX4353 and OX5036 effectors indicated that either the inclusion of the SG4 linker was responsible, or sequence changes to the C-terminal end of Dmprot2 (OX5036 included the full Dmprot2 sequence and a TMA tripeptide not associated with Dmprot2 was removed; in OX4353, the C-terminal lysine of Dmprot2 was removed and the TMA tripeptide included). However, it could not be excluded that the removal of Dmprot2

introns or the inclusion of a T2A-nls-zsGreen-nls fragment associated with the tetracycline-repressible transcriptional activator (tTAV) were responsible. Therefore, we decided to use a version of the Dmprot2-FokI effector more similar to that of OX4353, in future applications (**Chapter 5**).

In terms of fluorescent sperm marking, neither system (OX4718 or OX5036) was suitable for purpose. We observed weak fluorescence in most spermatids of OX4718A-resolved males (Dmprot2-mCherry-FokI), but it was not possible to reliably identify fluorescently labelled sperm, post-individualisation. This was not surprising, as an equivalent olive fly construct (OX4705: tetO21-Dmprot2-mCherry-FokI) demonstrated no marking (Asadi, 2013). The OX5036 system (Ccβ2tubulin[short]-Cchsp83-tTAV-T2A-nls-zsGreen-nls), in which the fluorescent marker protein was translated from the same mRNA as tTAV, was essentially invisible in most sperm. It was therefore necessary to design an entirely new fluorescent sperm marking system, ideally constitutively expressed, and not associated with the male sterility effector or tetracycline-repressible transcriptional activator. Furthermore, it was necessary to characterise a repressible male sterility effector with later translation. This was anticipated to require removal of the Dmhsp70 5'UTR and incorporation of a 5'UTR capable of imposing the appropriate translational delay. To achieve these aims, we selected systems based on two protamine-like genes of Medfly (Ccprot1 and Ccprot2) and another protamine-like gene from *D. melanogaster* (Dmprot1/Mst35Ba). As protamine-like genes are strongly and selectively localised to spermatids and sperm of *D. melanogaster*, this indicated that they could be applied for appropriate localisation of the male sterility and fluorescent sperm marking effectors in Medfly (Barckmann et al., 2013). Reporter constructs for these systems are evaluated in the next chapter, where the Medfly protamine-like genes were confirmed to possess desirable patterns of expression to engineer transgenic effectors for repressible male sterility and fluorescent sperm marking.

Chapter 4 – Novel regulators for effectors of repressible male sterility and a fluorescent sperm marking system to monitor mating ability in the field

4.1 Identification of protamine-like genes and expression construct design

The promoter, UTRs, and open reading frame of three protamine-like genes were selected as candidate regulatory systems of transgenic effectors in the male germline. Two sequences were selected from Medfly (Ccprot1 and Ccprot2) and one from *D. melanogaster* (Dmprot1). *D. melanogaster* protamine-like genes (Dmprot1 and Dmprot2) were identified from literature reports (Jayaramaiah Raja and Renkawitz-Pohl, 2005), (Tirmarche et al., 2014). Notably, Dmprot2 protein (almost identical to Dmprot1) was previously tested in OX4353, OX4718 and OX5036, with a different conformation (under transcriptional regulation of the tetracycline-repressible male germline expression system [tetO21-Dmhsp70 minipromoter-Dmprot2-FokI], rather than the protamine promoter. The protamine family is rapidly diverging, despite a high degree of functional conservation and an important role in male sexual development (Queralt et al., 1995), (Braun, 2001). As previously discussed (**Section 1.4.2.2**), they are typically small, basic, positively charged and contain high-mobility group (HMG) box motifs for DNA binding; this facilitated computational identification of Medfly protamine-like genes (Ccprot1 and Ccprot2) by Tarig Dafa'alla (**Figure 4.1**). A similarity index was calculated between sequences of interest, as the ratio of conserved residues (identical or conservative substitutions) to the mean number of residues. As expected, Medfly protamine-like genes were not very similar to *D. melanogaster*; the species diverged about 100 million years ago (Wulbeck and Simpson, 2000). Conservation was mostly limited to the DNA-binding domains (HMG motifs) and C-termini.

A

```
Ccprot1 (OX5122/3)            167 residues
MEVQKTFSSRSTTKPKGNSKTAFYRTKTGKCQRPSGILLRNPYLNFLREFRVRNTGLSAVEIIRRGAKEWNNMPKE
DKLHYIEEAFHAPKKRKPPSIQIQPQPNQMCAMPYQSDRGIVCPRMPTACPKKRRRKAKCARRRKPKKRRCGKKRR
PKRRVCKPRRKRCKI

Ccprot2 (OX5150)              215 residues
MGHCMRGGPITSNGYLNFLREFRKKCCGLSAVETVRQGAKLWNRLSCKQKDKYRLMGRNARPRRRRCAPKRKRRSC
APRRRKRRSCAPRRRKRRSCARKRRRPRCGKRRRPKRRRSCRPKRSRPRRRPRGCSRRRKRCMKPGPVTANAFLNF
LRAYRRKHCGLTPQETVKKGARRWCSLSPECKRRYMRQACKMSKSKRKKRSRICRSFHKKRRC

Dmprot1/Mst35BA (OX5140)      146 residues
MSSNNVNECKSLWNGIISISAKDESPKGLTEMCNHPIRRAPQKCKPMKSCAKPRRKAACAKATRPKVKCAPRQKCS
KQGPVTNNAYLNFVRSFRKKHCNLKPRELIAKAAKAWARLSENRKDRYRRMACKVTTSERHKRRRICQQY

Dmprot2/Mst35BB (OX4718/5036) 144 residues
MSSNNVNECKSLWNGIISISAKDESPKGLTEMCNHPKRRAPPKCKPMKSCAKPRRKAACAKATRPKVKCAPSQKCS
KQGPVTNNAYLNFVRFFRKKHCDLKPQELIAEAAKAWAELPEHRKDRYRRMACKVTTSERHKRRRICK
```

B

```
Ccprot1       1 --------RNPYLNFLREFRVRNTGLSAVEIIRRGAKEWNNMPKEDKLHYIEEA--
Ccprot2-A     1 ---------NGYLNFLREFRKKCCGLSAVETVRQGAKLWNRLSCKQKDKYRL-M--
Ccprot2-B     1 CMKPGPVTANAFLNFLRAYRRKHCGLTPQETVKKGARRWCSLSPECKRRYMRQACK
Dmprot1       1 --------NNAYLNFVRSFRKKHCNLKPRELIAKAAKAWARLSENRKDRYRRMACK
Dmprot2       1 --------NNAYLNFVRFFRKKHCDLKPQELIAEAAKAWAELPEHRKDRYRRMACK
```

C

```
Ccprot1     1 MEV-----------------------------QKTFS-----------------------
Ccprot2     1 MGHCMRGGPITSNGYLNFLREFRKKCCGLSAVETVRQGAKLWNRL-SCKQKDKYRLMGRN
Dmprot1     1 MSS-----------------------------NNVNECKSLWNGIISISAKDE-------
Dmprot2     1 MSS-----------------------------NNVNECKSLWNGIISISAKDE-------

Ccprot1     9 ---------------------------------SRSTTKPKGNSKTAFYRTK-------
Ccprot2    60 ARPRRRRCAPKRKRRSCA-PRRKRRSCAPRRRKRRSCARKRRRPRCGKKRRPKRRRSCR
Dmprot1    25 --------SPKGLTEMCNHPIRRAPQKCKP----MKSCAKPRRKAACAKATRPKVK--CA
Dmprot2    25 --------SPKGLTEMCNHPKRRAPPKCKP----MKSCAKPRRKAACAKATRPKVK--CA

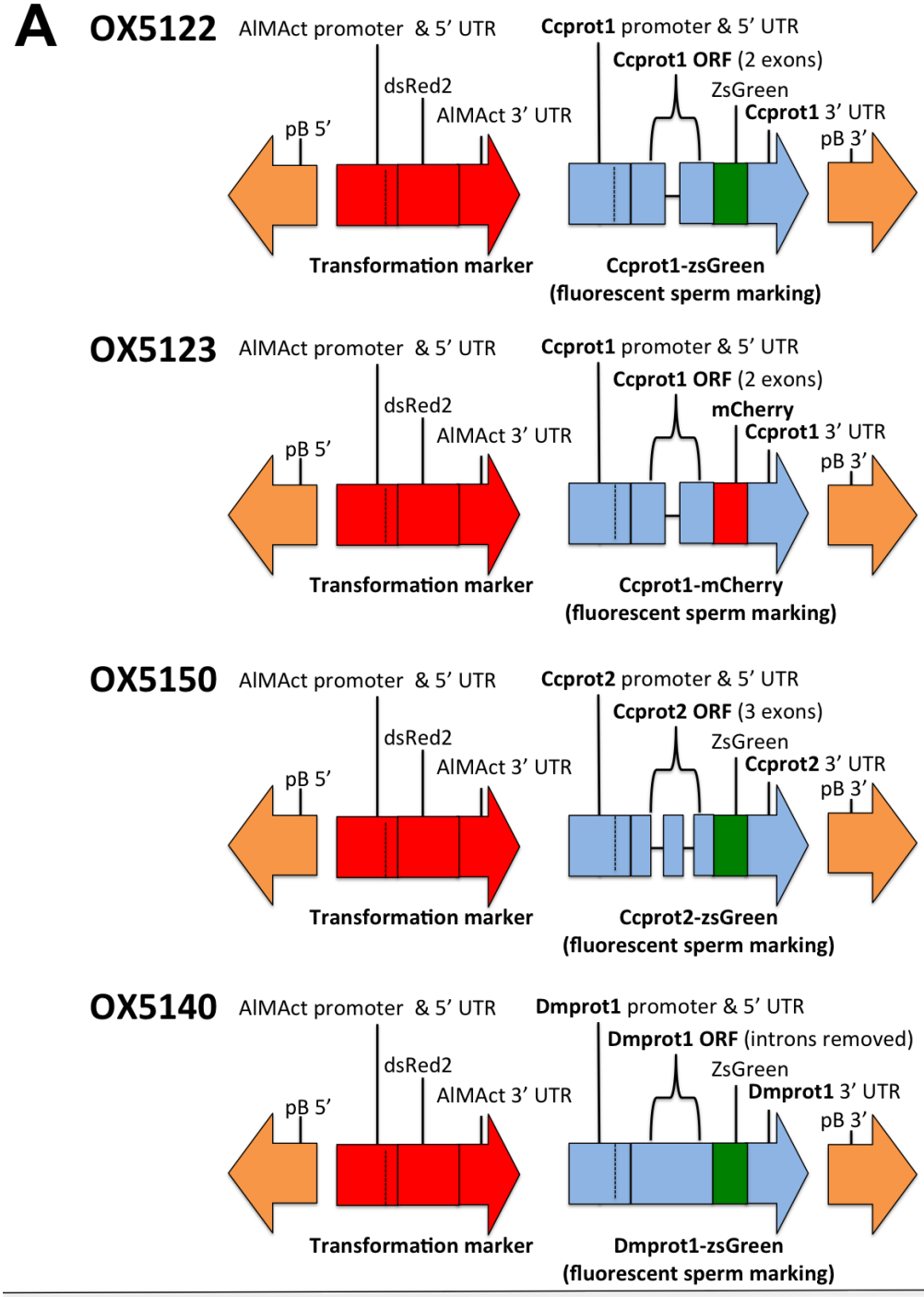
Ccprot1    28 ----------------TGKCQRPSGILLRNPYLNFLREFRVRNTGLSAVEIIRRGAKEWN
Ccprot2   119 PKRSRPRRRPRGCSRRRKRCMK-PGPVTANAFLNFLRAYRRKHCGLTPQETVKKGARRWC
Dmprot1    71 P---------------RQKCSK-QGPVTNNAYLNFVRSFRKKHCNLKPRELIAKAAKAWA
Dmprot2    71 P---------------SQKCSK-QGPVTNNAYLNFVRFFRKKHCDLKPQELIAEAAKAWA

Ccprot1    72 NMPKEDKLHYIEEAFHAPKKRKPPSIQIQPQPNQMCAMPYQSDRGIVCPRMPTACPKKRR
Ccprot2   178 SLSPECKRRYMRQACKM-----------------------------------------
Dmprot1   115 RLSENRKDRYRRMACKV-----------------------------------------
Dmprot2   115 ELPEHRKDRYRRMACKV-----------------------------------------

Ccprot1   132 RKAKCARRRKPKKRRCGKKRRPKRRVCKPR--RKRCKI
Ccprot2   195 ---------------SKSKRKKRSRICRSFHKKRRC--
Dmprot1   132 ---------------TTSERHKRRRICQQY--------
Dmprot2   132 ---------------TTSERHKRRRICK----------
```

Figure 4.1. Analysis of protamine-like genes applied in this study. (**A**) Full sequences of Medfly (Cc) and *D. melanogaster* (Dm) protamines with HMG (high mobility group) DNA binding motifs indicated (boldface). Ccprot2 has two motifs; Dmprot1, Dmprot2 and Ccprot1 have one. (**B-C**) Sequence alignments of HMG domains (**B**) and whole protamine sequences (**C**). Conserved residues shown in black and conservative substitutions in grey. HMG domains of the four protamines are highly conserved within and between species. The Dm protamine-like genes are essentially identical (similarity index: 96.6%). The Cc protamines are far less conserved (similarity index: 47.1%), indicating that their duplication and divergence in Medfly is more ancient.

We thereafter tested the ability of regulatory systems based on Ccprot1, Ccprot2 and Dmprot1 to localise expression of a fluorescent reporter (zsGreen or mCherry) to the male germline (**Figure 4.2**): the zsGreen or mCherry ORF was cloned as a C-terminal fusion with the protamine ORF, to reveal protein production and localisation (protamine promoter-protamine 5'UTR-protamine ORF-zsGreen/mCherry-protamine 3'UTR). The Ccprot1 and Ccprot2 ORFs incorporated the native introns; they were removed in Dmprot1. The ideal phenotype would demonstrate reproducible expression (not insertion sensitive) of the fluorescent reporter, with robust localisation to the nucleus of sperm and spermatids, but not spermatocytes. This would indicate that an element of the regulatory system mediated translational delay. The protamine-like genes of *D. melanogaster* are known to be translationally repressed by sequences within the 5'UTR; it was therefore likely that Medfly protamine-like genes were equivalently regulated (Jayaramaiah Raja and Renkawitz-Pohl, 2005). As previously discussed, it would be preferable to delay the localisation of protamine-FokI to elongating spermatids; early translation would be anticipated to disrupt the meiotic divisions or cause significant defects in spermatid nuclear shaping, leading to failure of spermatid individualisation.



B

```
OX5122 (Ccprot1-zsGreen) KRCKIRYRSTMAQSK
OX5123 (Ccprot1-mCherry) KRCKIRYRYVSKGEE
OX5150 (Ccprot2-zsGreen) KKRRCRYRSTMAQSK
OX5140 (Dmprot1-zsGreen) ICQQYRYRSTMAQSK
```

Figure 4.2. Evaluation of protamine-based regulators for repressible male sterility and fluorescent sperm marking, by reporter expression in the male germline. (**A**) Three regulatory systems were tested: Ccprot1 (OX5122/5123), Ccprot2 (OX5150) and Dmprot1 (OX5140). The brightness of two reporters for fluorescent sperm marking (zsGreen and mCherry) was compared with an identical Ccprot1 driver in OX5122 (Ccprot1-**zsGreen**) and OX5123 (Ccprot1-**mCherry**). All constructs featured the previously described somatic transformation marker (AlMAct-dsRed2) and unused sites for site-specific recombination (not shown), which were artefacts of the prior vector backbone: upstream of AlMact-dsRed2 (loxp, frt3, frt, attp58) and downstream of the protamine-reporter (attp220). Dashes indicate promoter-5'UTR boundary. (**B**) Alignment of sequences at the protamine-fluorescent marker fusion sites. All fusions incorporated a short linker (boldface) between protamine and the fluorescent marker (underlined). Note that OX5123 omits the N-terminal methionine of mCherry.

It was also essential that there would be no effect on male mating competitiveness, associated with expression of the reporter in the male germline. Furthermore, the fluorescent marking system needed to be applicable to field study, demonstrating successful transfer of marked, motile sperm to females with detectable fluorescence persisting at least one week after death of the mated female (to assess mating competitiveness in the field). Functioning candidates were to be used in the design of a new generation of all-in-one constructs for repressible male sterility and fluorescent sperm marking. Modification of the protamine-reporter fragment would thereafter be required to generate a male sterility effector (**Section 4.5**).

4.2 Evaluation of Ccprot1 (OX5122/OX5123), Ccprot2 (OX5150), and Dmprot1 (OX5140) constructs expressing fluorescent reporters in the male germline

4.2.1 Establishment of OX5122, OX5123, OX5140 and OX5150 transgenic lines

Microinjection (**Table 4.1**) and backcrossing to WT (**Table 4.2**) were performed as previously described (**Chapter 2)**, using *piggyBac* helper OX3022 (300 ng/µl) and one of four expression constructs OX5122, OX5123, OX5140 or OX5150 (600 ng/µl). Each construct was injected independently. To verify successful microinjection, surviving G_0 pupae were screened for transient expression of the AlMAct-dsRed2 transformation marker. Adult survival was greater than the average rate for Medfly microinjections at Oxitec (25%), for OX5122 and OX5123, and less for OX5140 and OX5150 (Gregory et al., 2016). In general, four lines were kept for each construct for initial phenotypic analysis (three for OX5150; one line failed to propagate).

From each pool, one transgenic G_1 male was outcrossed to three WT females and eggs collected five days later to establish a transgenic line. As previously described, each line was assessed for Mendelian characteristics: insertion copy number (transgenic to wildtype ratio) and sex-linkage (sex distribution of transgenic progeny). In certain instances, surplus male transgenic G_1 individuals were immediately dissected, to provide a preliminary analysis of the performance of the transgenic regulator one generation earlier (with the caveat that different insertions may have been assessed within each pool, because molecular genotyping of insertions was not performed on these individuals).

Table 4.1: Microinjection logistics for OX5122, OX5123, OX5140 & OX5150

Construct	Embryos	Larvae	Pupae	Adults	Lines
OX5122	963	451 (47%)	350 (36%)	348 (36%)	11 (3%)
OX5123	766	376 (49%)	318 (42%)	310 (40%)	17 (5%)
OX5140	1339	398 (30%)	353 (26%)	232 (17%)	8 (3%)
OX5150	466	111 (24%)	93 (20%)	79 (17%)	7 (9%)

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (transgenic lines/G_0 adults crossed).

Table 4.2: G_0 backcrosses to establish OX5122, OX5123, OX5140 & OX5150 lines

OX5122A	**30 ♂ x 90 WT ♀**	**OX5122B**	**30 ♂ x 90 WT ♀**	OX5122C	20 ♀ x 20 WT ♂
OX5122D	**20 ♀ x 20 WT ♂**	**OX5122E**	**30 ♂ x 90 WT ♀**	**OX5122F**	**20 ♀ x 20 WT ♂**
OX5122G	**30 ♂ x 90 WT ♀**	**OX5122H**	**20 ♀ x 20 WT ♂**	OX5122I	20 ♀ x 20 WT ♂
OX5122J	30 ♂ x 90 WT ♀	**OX5122K**	**15 ♀ x 15 WT ♂**	**OX5122L**	**21 ♂ x 21 WT ♀**
OX5122M	**24 ♀ x 16 WT ♂**	**OX5122N**	**24 ♂ x 16 WT ♀**	OX5122O	11 ♂ x 13 WT ♀
OX5123A	**30 ♂ x 90 WT ♀**	**OX5123B**	**10 ♂ x 25 WT ♀**	**OX5123C**	**20 ♀ x 10 WT ♂**
OX5123D	**20 ♀ x 10 WT ♂**	OX5123E	10 ♂ x 25 WT ♀	**OX5123F**	**11 ♂ x 25 WT ♀**
OX5123G	**10 ♂ x 25 WT ♀**	OX5123H	10 ♂ x 25 WT ♀	OX5123I	10 ♂ x 25 WT ♀
OX5123J	**10 ♂ x 25 WT ♀**	**OX5123K**	**10 ♂ x 25 WT ♀**	**OX5123L**	**10 ♂ x 25 WT ♀**
OX5123M	10 ♂ x 25 WT ♀	**OX5123N**	**10 ♂ x 25 WT ♀**	**OX5123O**	**10 ♂ x 25 WT ♀**
OX5123P	**20 ♀ x 10 WT ♂**	**OX5123Q**	**20 ♀ x 10 WT ♂**	**OX5123R**	**20 ♀ x 10 WT ♂**
OX5123S	20 ♀ x 10 WT ♂	**OX5123T**	**20 ♀ x 10 WT ♂**	**OX5123U**	**14 ♀ x 10 WT ♂**
OX5123V	**8 ♂ x 7 ♀**				
OX5140A	15 ♂ x 25 WT ♀	**OX5140B**	**15 ♂ x 25 WT ♀**	OX5140C	15 ♂ x 25 WT ♀
OX5140D	15 ♂ x 25 WT ♀	**OX5140E**	**15 ♂ x 25 WT ♀**	**OX5140F**	**15 ♂ x 25 WT ♀**
OX5140G	8 ♂ x 25 WT ♀	**OX5140H**	**25 ♀ x 10 WT ♂**	**OX5140I**	**25 ♀ x 10 WT ♂**
OX5140J	24 ♀ x 10 WT ♂	**OX5140K**	**15 ♂ x 25 WT ♀**	**OX5140L**	**25 ♀ x 10 WT ♂**
OX5140M	**20 ♀ x 10 WT ♂**				
OX5150A	**8 ♀ x 8 WT ♂**	**OX5150B**	**10 ♀ x 10 WT ♂**	**OX5150C**	**10 ♀ x 10 WT ♂**
OX5150D	**10 ♀ x 10 WT ♂**	OX5150E	10 ♂ x 30 WT ♀	**OX5150F**	**10 ♂ x 30 WT ♀**
OX5150G	**9 ♂ x 30 WT ♀**	**OX5150H**	**8 ♂ x 30 WT ♀**		

Underlined pools yielded transgenics. Permanent transgenic lines were established from **OX5122**D, E, G, K & M; **OX5123**C, D, G, J & U; **OX5140**E, F, K, L; and **OX5150**B, F and H. Additional individuals were assessed at the G_1 stage without molecular genotyping or the establishment of permanent lines, from pools **OX5140**E, F, K & L; and **OX5150**B, C, F & H.

4.2.2 The Ccprot1 regulatory system successfully mediates zsGreen (OX5122) or mCherry (OX5123) expression in the male germline

The fluorescent proteins zsGreen (OX5122) and mCherry (OX5123) were evaluated under regulatory control of an identical Ccprot1 system (Ccprot1 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen/mCherry-Ccprot1 3'UTR**)** to determine which reporter provided the strongest sperm marking. Five stable lines were established for each construct (**Table 4.3**).

Table 4.3: Mendelian analysis of OX5122 and OX5123 transgenic lines

Line	**Sex**	**Copy number**		**Sex-linkage**		**Summary**		**Fluorescence in testes**
		Transgenic (%)	n	Sex ratio (M/F)	n	Copies	Location	
OX5122D	♂	54	353	1.3	177	1	Autosome	Yes
OX5122E	♂	49	255	1.2	101	1	Autosome	Yes
OX5122G	♂	54	229	1.7	101	1	Autosome	Yes
OX5122K	♂	60	125	1.1	51	1	Autosome	Yes
OX5122M	♂	48	182	2.1	66	1	Autosome	Yes
OX5123C	♂	51	227	0.6	94	1	Autosome	Yes
OX5123D	♂	50	304	1.5	131	1	Autosome	Yes
OX5123U	♂	**77**	220	1.0	143	**2**	Autosome	Yes
OX5123G	♂	**74**	257	2.0	163	**2**	Autosome	**No**
OX5123J	♂	**78**	300	2.4	176	**2**	Autosome	**No**

All lines except OX5123G, U and J appear to be double insertions. At least one of the insertions of OX5123G, U and J appear to be autosomal. All other lines were single, autosomal insertions. "N" refers to number screened. Localisation of the fluorescent reporter in testes was assessed in the subsequent sections.

Heterozygous G_2 OX5122 (Ccprot1-zsGreen) and OX5123 (Ccprot1-mCherry) males (n=20) were collected on eclosion. Strong fluorescence was seen in spermatids and sperm, but not spermatocytes, of all OX5122 lines and 3/5 OX5123 heterozygous lines (**Figure 4.3)**.

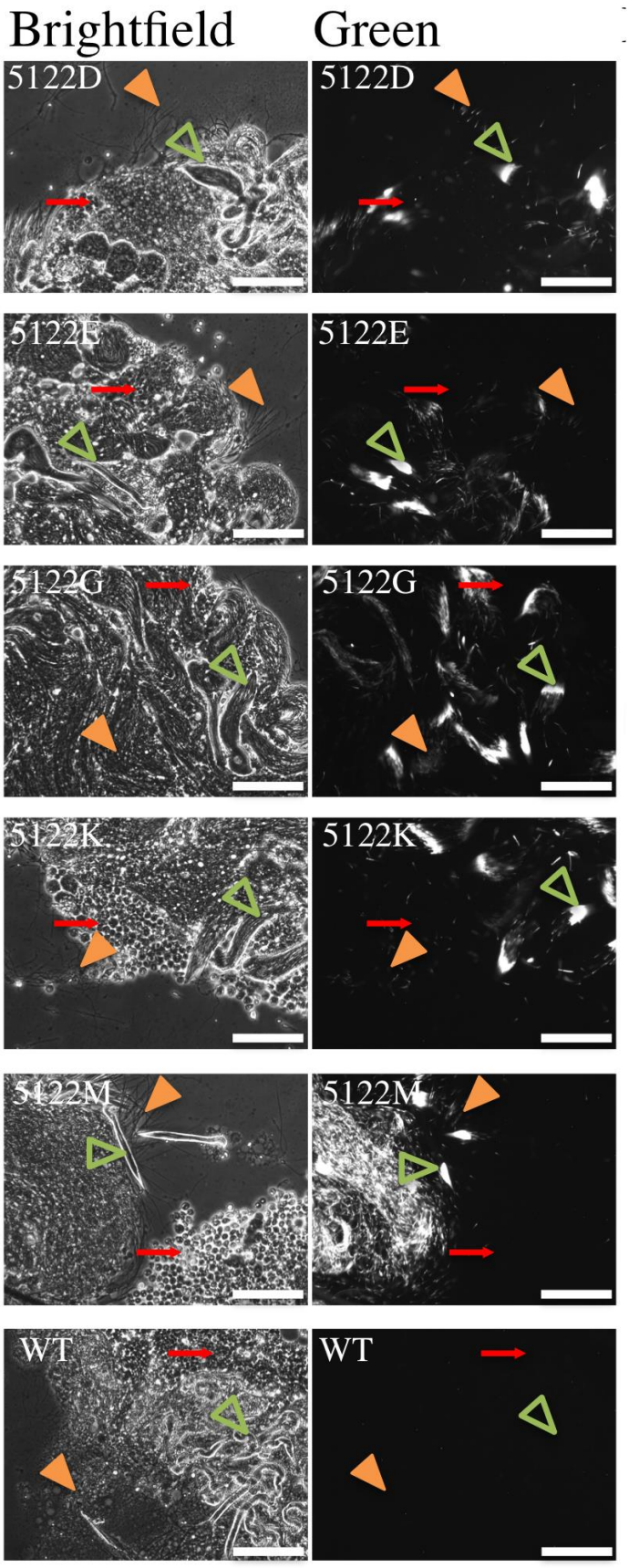


Figure 4.3. The Ccprot1 regulatory system localises a reporter to spermatids and sperm of all five heterozygous OX5122 (Ccprot1-ZsGreen) lines, and three of five OX5123 (Ccprot1-mCherry) lines (continued on next page). Fluorescence and phase contrast microscopy of dissected Medfly testes demonstrate that sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), are brightly marked green in OX5122 lines and red in certain OX5123 lines. Scale bars: 100 μm.

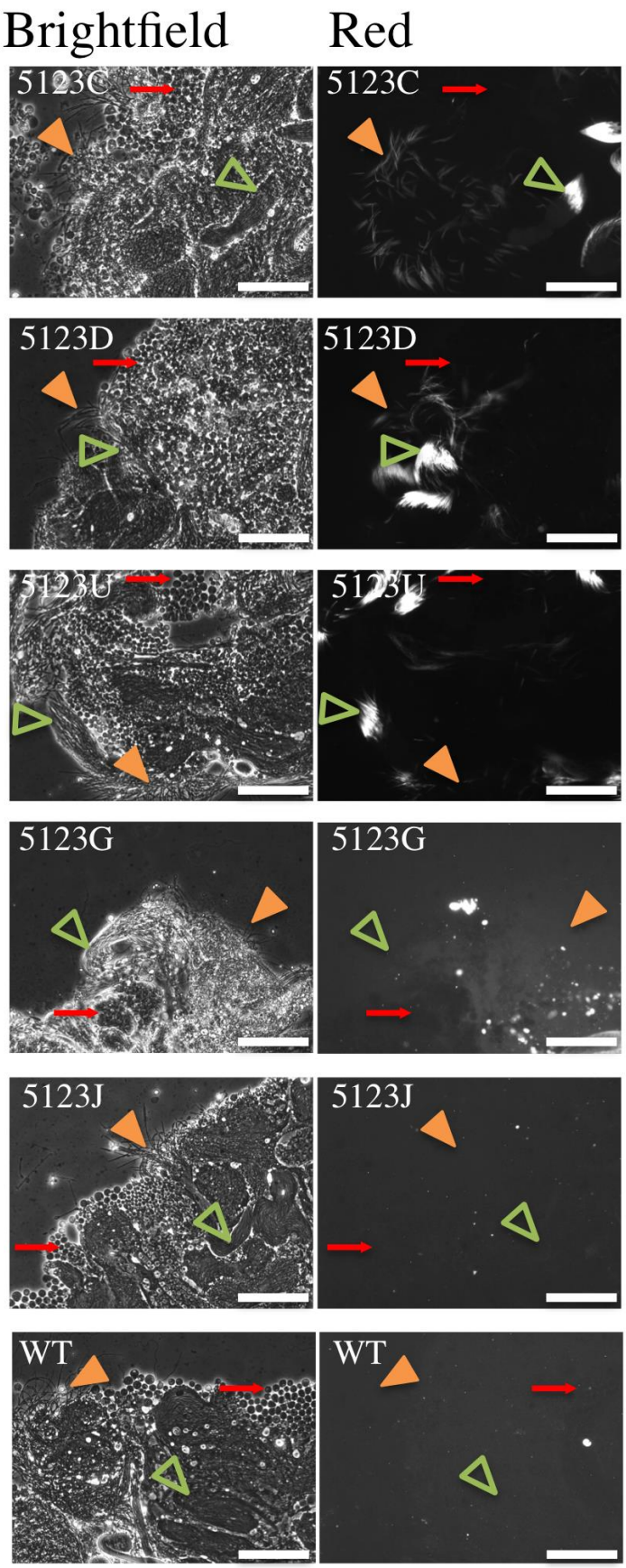


Figure 4.3. The Ccprot1 regulatory system localises a reporter to spermatids and sperm of all five heterozygous OX5122 (Ccprot1-ZsGreen) lines, and three of five OX5123 (Ccprot1-mCherry) lines. Fluorescence and phase contrast microscopy of dissected Medfly testes demonstrate that sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), are brightly marked green in OX5122 lines and red in certain OX5123 lines. Scale bars: 100 µm.

This indicated post-meiotic translation consistent with the desired expression profile; the nuclear localisation of the marker was also ideal. Marking in OX5122 (Ccprot1-zsGreen) lines appeared less sensitive to insertion site: all five OX5122 lines were brightly marked, but two of five OX5123 lines were not visibly marked. We therefore concluded that Ccprot1 is a strong candidate system to regulate effectors of repressible male sterility; and that the Ccprot1-zsGreen fluorescent sperm marking system (OX5122) outperformed the Ccprot1-mCherry system (OX5123). As the Ccprot1 system was to be applied extensively in future product candidates, we decided to investigate potential effects on mating competitiveness and fluorescent marker persistence in sperm transferred to mated females. These results are presented (**Section 4.3**), after the assessments of fluorescent reporter localisation in the other systems (OX5150 [Ccprot2-zsGreen] and OX5140 [Dmprot1-zsGreen]).

4.2.3 The Ccprot2 regulatory system (OX5150) successfully mediates zsGreen expression in the male germline

The ability of the Ccprot1 regulatory system (OX5122/5123) to localise a protamine-reporter fusion in the male germline, led us to expect a similar result for Ccprot2 (OX5150), because the *D. melanogaster* homologues have overlapping expression and function (Jayaramaiah Raja and Renkawitz-Pohl, 2005). The construct was evaluated essentially as described for OX5122, with exceptions noted below. Transgenic lines were established from single G_1 individuals of three of four pools (OX5150B, F and H; OX5150C died pre-establishment). The remaining male transgenic G_1 individuals (n = 21) from these four pools were immediately dissected (without establishing lines), as a preliminary analysis of performance of the Ccprot2-zsGreen system (with the caveat that individuals of the same pool were likely to demonstrate the same insertion, in some instances). No fluorescence was observed in the testes of most individuals (16/21). Results are summarised in **Table 4.4** and **Figure 4.4**.

Table 4.4: Ccprot2-zsGreen expression in transgenic OX5150 G_1 males

Pool	Individuals expressing Ccprot2-zsGreen in testes	Conclusion
OX5150B	2/5	≥ 2 lines assessed
OX5150C	2/3	≥ 2 lines assessed
OX5150F	1/10	≥ 2 lines assessed
OX5150H	0/3	≥ 1 lines assessed
Summary	*5/21*	*≥ 7 lines assessed*

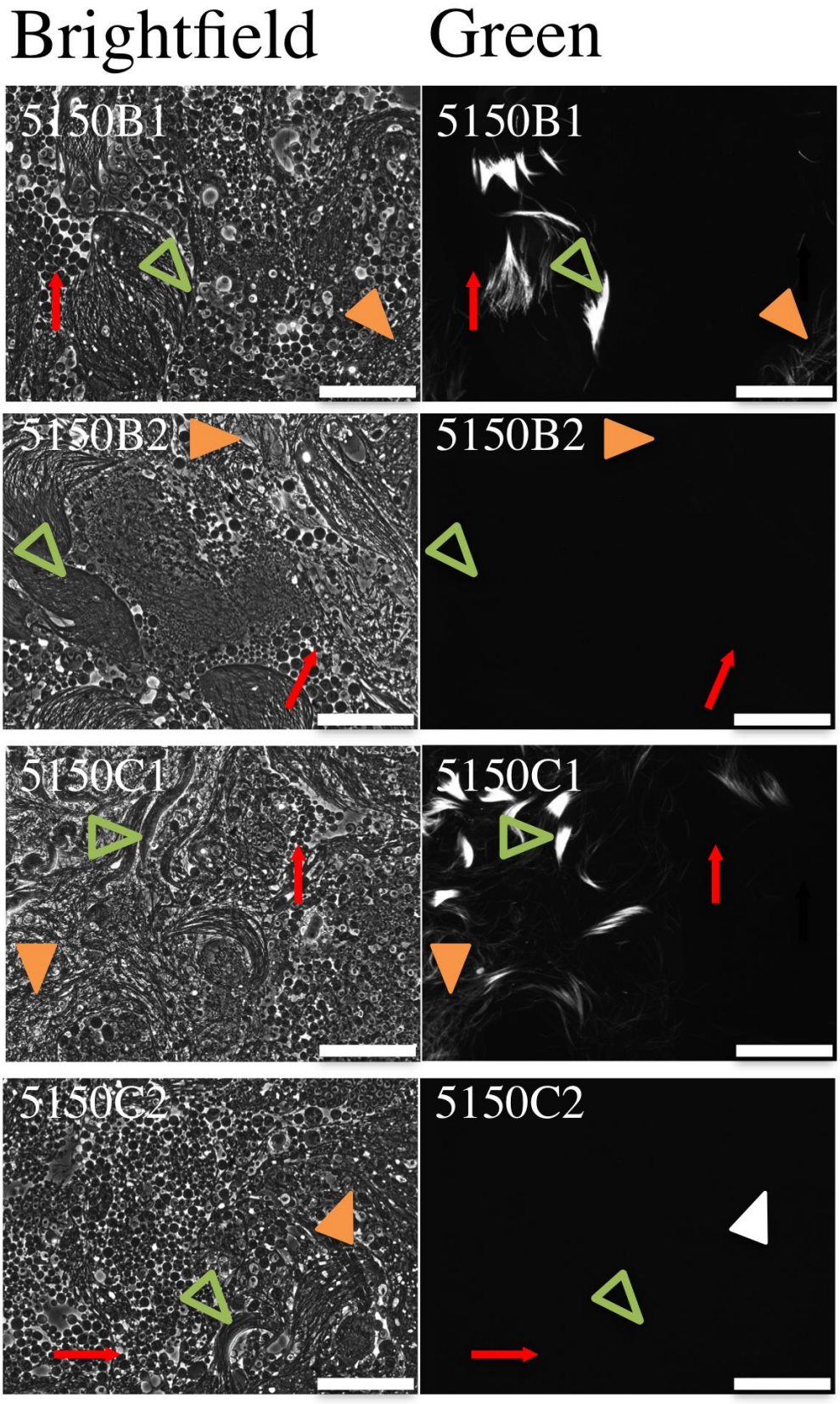
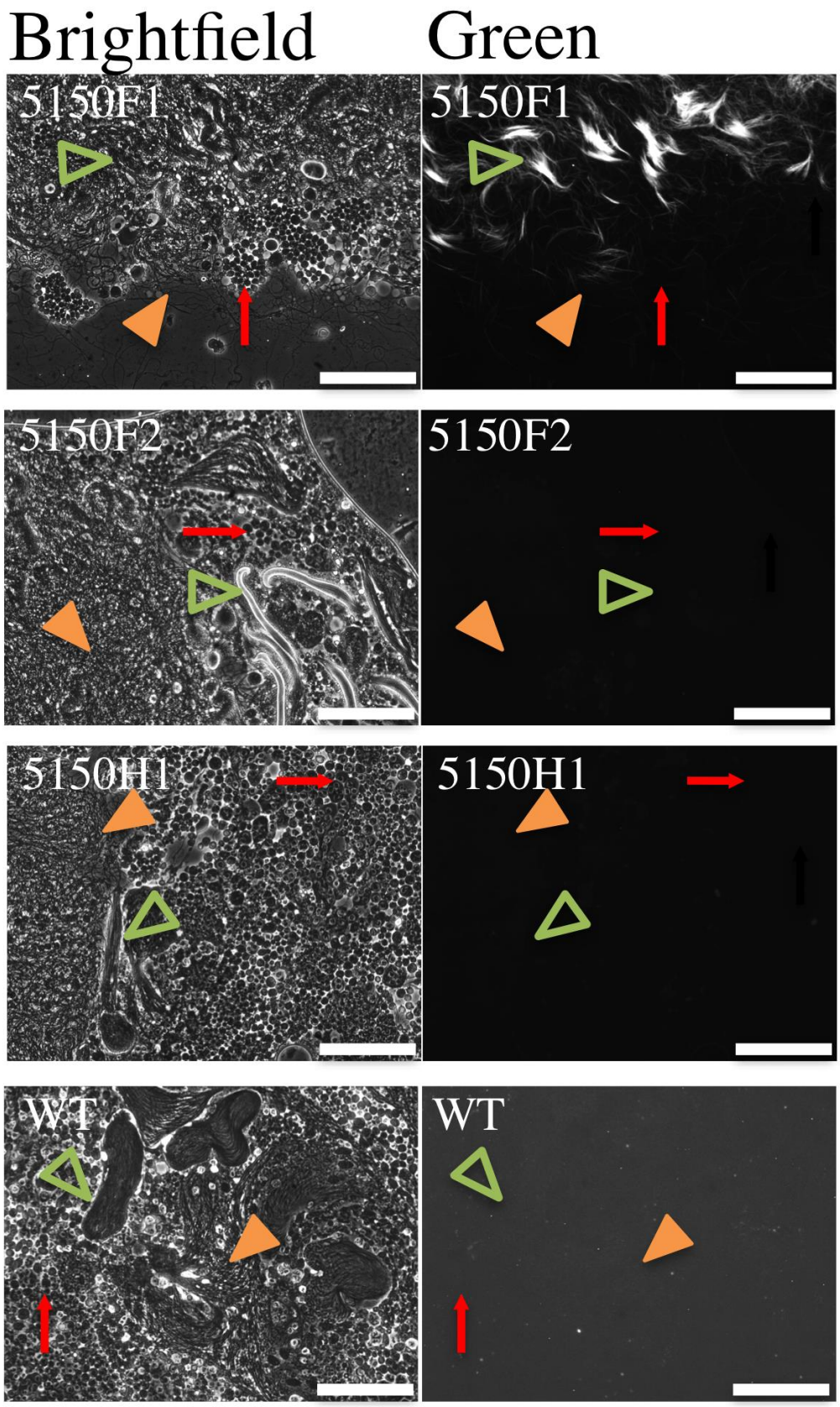


Figure 4.4. The Ccprot2 regulatory system (OX5150) appears insertion sensitive and does not localise Ccprot2-zsGreen to spermatids and sperm of all transgenic lines (continued on next page). Fluorescence and phase contrast microscopy of dissected Medfly testes from individuals of seven apparent lines, from three G_1 transgenic pools. Marking is present in sperm (**shaded arrowhead**) and spermatids (**unshaded arrowhead**), but not spermatocytes (**arrow**) of individuals OX5150B1, C1, and F1. Individuals OX5150B2, C2, F2 and H1 completely lacked expression. Scale bars: 100 μm.



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The remaining individuals (5/21) demonstrated bright fluorescence in their testes, which did not appear noticeably different to Ccprot1-zsGreen (OX5122). Three of four pools (OX5150B, C, F) included males with and without fluorescent reporter localisation in testes; all individuals of OX5150H (n=3) lacked visible fluorescence. As molecular genotyping was not performed, it was not possible to determine if individuals of the same pool that demonstrated equivalent phenotypes, shared the same insertion. Therefore, it appears that at least two independent insertions were assessed from OX5150B, C & F (reporter localisation observed in certain, but not all, individuals); and at least one from OX5150H (reporter localisation not observed in any individuals). This assumes that a particular insertion results in a similar phenotype across all individuals, which was observed for all ten OX5122/OX5123 lines.

The established transgenic lines (OX5150B3, OX5150F3 & OX5150H2) were all single insertions (**Table 4.5**); two were y-linked and one was autosomal. OX5150C3 failed to propagate. These were assessed in a similar manner to OX5122, but scored for germline fluorescence on the Oxitec imaging setup at 10x magnification. It was not equipped with a camera at this stage, so imaging was not possible. The observed phenotype was consistent across all individuals of a particular line. zsGreen did not localise to the germline for individuals of two lines (OX5150F3 & H2); localisation was observed in individuals of one line (OX5150B3). This analysis did not provide further information regarding the functionality of Ccprot2-zsGreen, because negative individuals had already been identified from OX5150F and OX5150H transgenic pools, and positive individuals from OX5150B, in the prior generation. As these previously noted phenotypes were not linked to a particular insertion, it is likely that OX5150B3, F3 and H2 were equivalent respectively to OX5150B1, F2 and H1. It was not deemed necessary to repeat the experiment to obtain images for these three redundant phenotypes.

Table 4.5: Mendelian analysis of OX5150 transgenic lines

Line	**Sex**	**Copy number**		**Sex-linkage**		**Summary**		**Fluorescence in testes**
		Transgenic (%)	n	Sexes observed	n	Copies	Location	
OX5150B3	♂	44	90	Both	> 20	1	Autosome	Yes
OX5150F3	♂	50	167	**Male-only**	> 20	1	**Y**	**No**
OX5150H2	♂	50	72	**Male-only**	> 20	1	**Y**	**No**

All lines are single insertions. OX5150F3 and H3 are Y-linked, which is likely to have explained why fluorescent reporter expression was not observed. “N” refers to number screened.

That we frequently observed a lack of fluorescence in the male germline of OX5150 individuals, indicated that the Ccprot2 (OX5150) promoter fragment was potentially more insertion-site sensitive than the Ccprot1 equivalent (OX5122/OX5123), where strong expression was observed in eight of ten autosomal insertions. However, both of the negative lines that were genotyped for insertion were y-linked (OX5150F3 and H2). This site is not typically favourable for male germline expression; we observed weak expression of transgenic effectors in the male germline, for y-linked insertions of other expression constructs (OX5036: **Section 3.7**; OX5257: **Section 5.5**). Individuals lacking fluorescent protein expression in testes, were observed during dissection of transgenic G_1 individuals from all four pools (OX5150B, C, F & H), but these individuals were not genotyped for insertion site. In retrospect, a superior analysis could have been performed by backcrossing all G_1 individuals, dissecting the testes after lines were established, and finally performing Mendelian analysis and further phenotypic assessment on those lines that demonstrated the phenotypes of interest.

4.2.4 The Dmprot1 regulatory system (OX5140) does not express zsGreen in the male germline despite detectable transcription

Analysis of OX5140 lines (Dmprot1-zsGreen) was essentially as described for OX5150. Transgenic lines were established from single individuals of four pools (OX5140E, F, K & L). As a preliminary analysis, testes of surplus male transgenic G_1 individuals (n=15) from these four pools, and three additional pools (OX5140B, H & I) were dissected (ie. at least seven insertions were assessed). Reporter expression was never detected, indicating that the Dmprot1 system (OX5140) would require modification to be developed into a useful expression system in Medfly. Although OX5140 appeared unsuitable for future use, it was necessary to exclude the possibility that poor expression resulted from insertion site (sex-linked insertions are frequently weak, as demonstrated in **Section 4.2.3**). It was also of interest, but not commercially essential, to investigate the source of failure; this would facilitate the future development of a modified, functional Dmprot1 system. We investigated four lines established from single individuals (OX5140E, F, K and L). All were single autosomal insertions (**Table 4.6**). Reporter expression in the male germline was assessed exactly as described for OX5122/OX5123 (ten pairs of testes dissected from each line at Cardiff University). Expression of the fluorescent reporter in testes was not observed in any of four lines (**Figure 4.5**), suggesting that lack of expression was not a consequence of insertion site, but rather was because of inherently poor performance of the construct. From these results, it was not possible to conclusively associate a lack of expression with the Dmprot1 promoter, untranslated regions or Dmprot1-zsGreen fusion.

Table 4.6: Mendelian analysis of OX5140 transgenic lines

Line	Sex	Copy number		Sex-linkage		Summary		Fluorescence in testes
		Transgenic (%)	n	Sexes observed	n	Copies	Location	
OX5140E	♂	46	362	Both	> 20	1	Autosome	No
OX5140F	♂	55	100	Both	> 20	1	Autosome	No
OX5140K	♂	50	254	Both	> 20	1	Autosome	No
OX5140L	♂	49	170	Both	> 20	1	Autosome	No

All lines were single autosomal insertions. “N” refers to number screened.

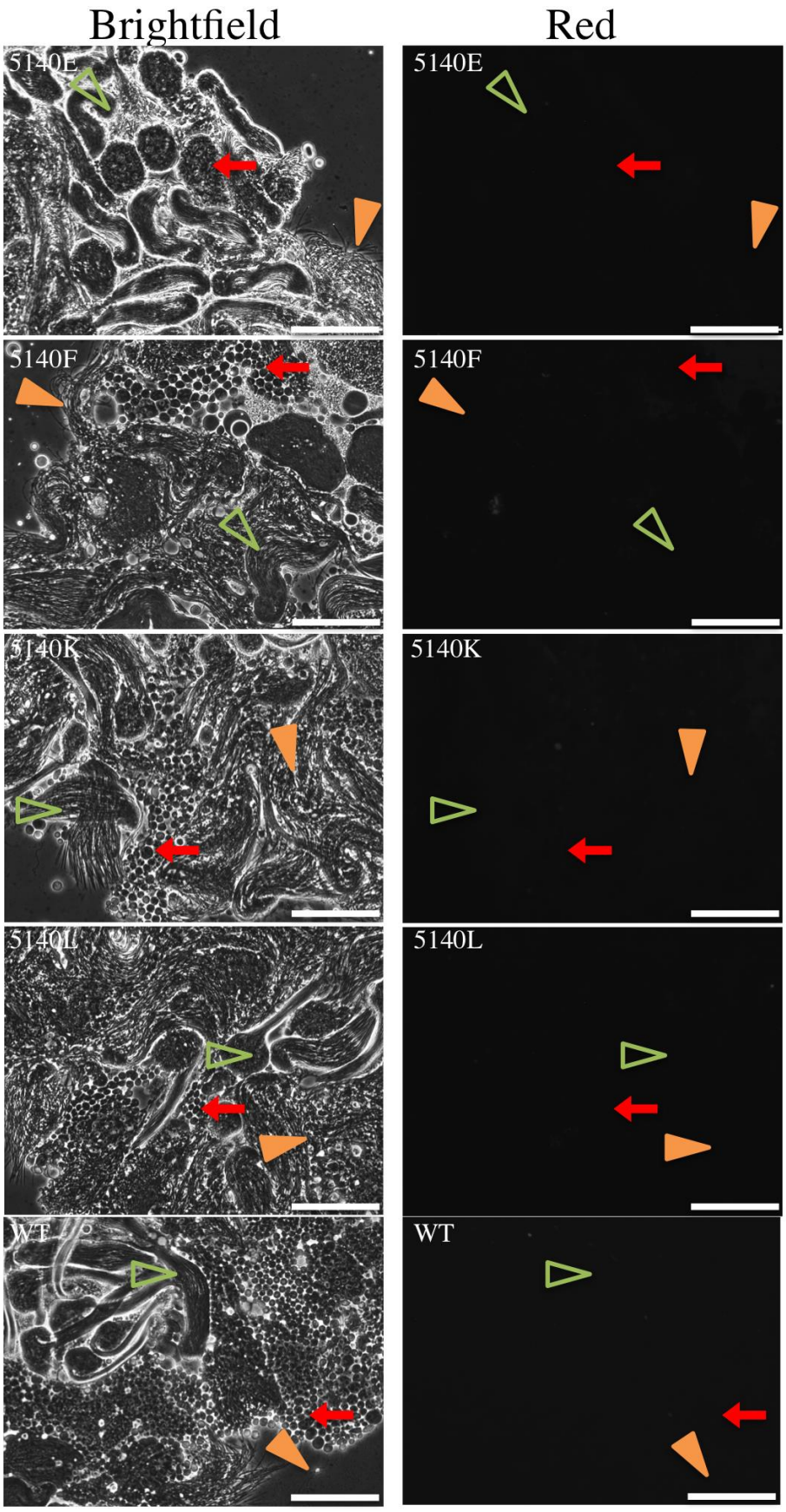


Figure 4.5. The Dmprot1 driver does not localise Dmprot1-zsGreen to spermatids and sperm of any of four heterozygous OX5140 lines evaluated. Fluorescence and phase contrast microscopy of dissected Medfly testes demonstrate that marking is absent in sperm (**shaded arrowhead**), spermatids (**unshaded arrowhead**) and spermatocytes (**arrow**). Scale bars: 100 μm.

RT-PCR was used to test whether Dmprot1-zsGreen transcript was produced in the transgenic lines. Failure to detect transcript would implicate the promoter, whereas the presence of transcript would implicate either the untranslated regions or the Dmprot1-zsGreen fusion. RNA was extracted from a single pair of testes of five OX5140E individuals. The expression of Dmprot1-zsGreen and an endogenous control (*adh*) was assessed. It was not possible to design primers differentiating cDNA and gDNA for Dmprot1-zsGreen, because the Dmprot1 ORF lacked introns. However, controls lacking reverse transcriptase were used, to identify potential gDNA contamination. The RT-PCR revealed that the Dmprot1-zsGreen mRNA was present in OX5140E testes; therefore the Dmprot1 promoter is capable of driving expression of a transgene in the Medfly testis **(Figure 4.6**). It appeared that the failure to observe reporter translation was associated with the 5'UTR or the Dmprot1-zsGreen fusion. The 3'UTR was excluded as a likely source of failure, because the Dmprot1 promoter-5'UTR fragment is sufficient to recapitulate endogenous expression, if provided a minimal 3'UTR (Jayaramaiah Raja and Renkawitz-Pohl, 2005).

We next considered if the coding sequence of Dmprot1 was not sufficient to mediate reporter localisation. This was unlikely because it is essentially identical to the Dmprot2 protein (97% conserved), which was able to localise Dmprot2-mCherry-FokI to DNA, in the male germline (OX4718, **Figure 3.5**). However, the fusion of Dmprot1 to ZsGreen may prevented its localisation to nuclear DNA; therefore it might not be detectable despite translation, because it was diffuse throughout the cell. It was alternatively possible that the fluorescence of zsGreen was blocked by the fusion. The Dmprot1-zsGreen fusion protein incorporated a short peptide (RYRST), preceding the fusion to zsGreen. It was not expected to have an effect, as it was present in Ccprot1-zsGreen and Ccprot2-zsGreen, both of which demonstrated extremely bright fluorescent reporter localisation. Interestingly, the homologous Dmprot2 protein was previously shown to poorly tolerate certain C-terminal fusions of Dmprot2 to mCherry, even when a short linker tripeptide (TMA) was present. For instance, fluorescent marker localisation in sperm of OX4801 (tetO21-Dmprot2-**SG4**-mCherry-**SG4**-FokI) and OX4705/4718 (tetO21-Dmprot2-mCherry-FokI) lines was frequently weak or absent (Asadi, 2013), (**Figure 3.3** & **Figure 3.5**). This suggests that the fusion is likely to be responsible for the poor translation of the fluorescent marker in the male germline, although it cannot be excluded that the Dmprot1 5'UTR does not mediate sufficient localisation in spermatids and sperm of Medfly.

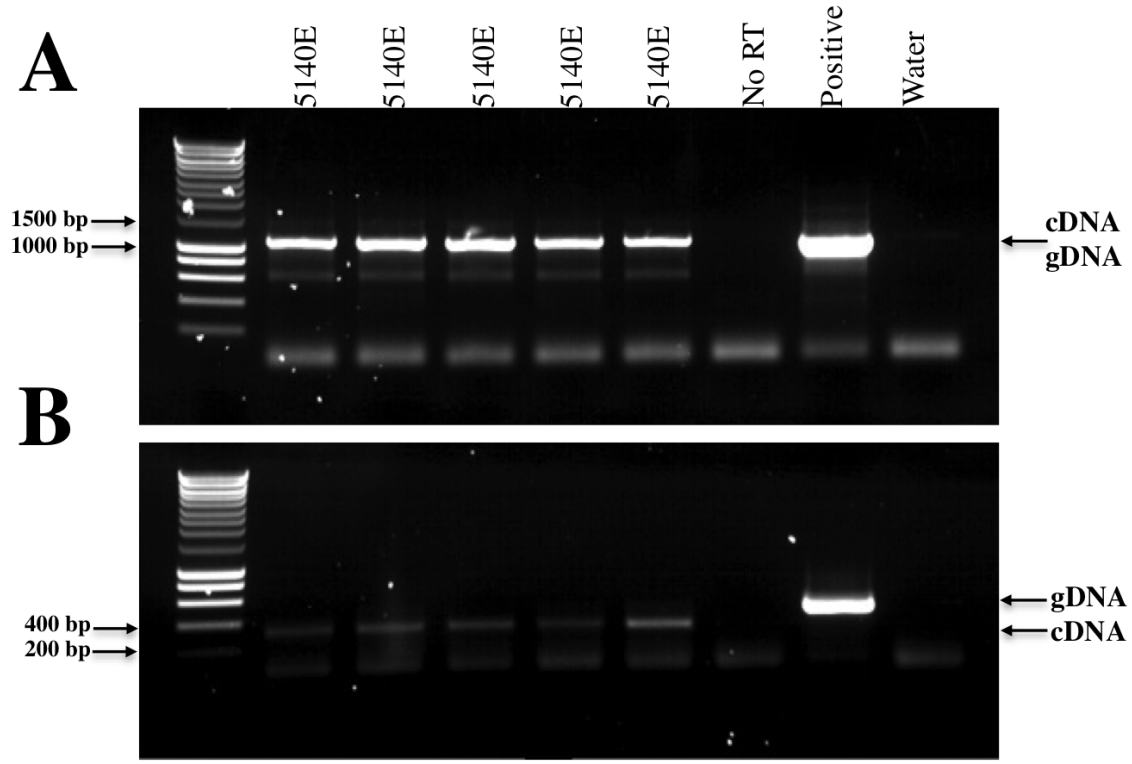


Figure 4.6. RT-PCR indicates that the Dmprot1-zsGreen reporter is transcribed, indicating that the Dmprot1 promoter fragment is functional. (**A**) ZsGreen RT-PCR (1043 bp [cDNA & gDNA]). (**B**) Endogenous (*adh*) PCR. (329 bp [cDNA]; 491 bp [gDNA]). Template was cDNA from a testis pair of five OX5140E males. Controls were reverse-transcriptase free (no RT, OX5140E #4), negative (water), and positive (OX5140E gDNA).

4.3 The Ccprot1-zsGreen (OX5122) fluorescent sperm marking system can be used to monitor the mating ability of transgenic males in the field

4.3.1 Ccprot1-zsGreen (OX5122) heterozygotes transfer fluorescently marked sperm and demonstrate mating competitiveness similar to WT

Functioning protamine-fluorescent marker fusions described in this chapter (OX5122, OX5123 or OX5150) were to be used without modification as fluorescent sperm marking systems, in future transgenic expression systems for commercial application in Medfly population control. For this reason, additional phenotypic aspects of the system that demonstrated best performance (OX5122 [Ccprot1-zsGreen]) were evaluated. We assessed four lines (OX5122D, G, K & M) for ability to transfer sperm, a crucial factor that was deficient in a previously characterised line that was otherwise promising as a commercial product (OX4718A, **Figure 3.8**). A single line (OX5122M) was subjected to further analysis (mating competitiveness, visibility of WT and transgenic sperm in a twice-mated female, and the persistence of the fluorescent sperm marker in females under field-like trapping conditions). The rationale was to gather

evidence that transgenic males could compete with wild males. Future product candidates (transgenic lines incorporating independent systems for fluorescent sperm marking and repressible male sterility) will require thorough analysis, prior to application to appropriate regulatory bodies for permission to conduct field trials and eventual commercial releases. Therefore, this initial investigation was intentionally limited in scope.

To assess the mating competiveness of OX5122M heterozygous males, three genotypes were collected on eclosion and reared independently in small cages (n=25 per cage): wild-type males, wild-type females and OX5122M heterozygous males (all reared off-tet). Five days after eclosion, wild-type and transgenic males (n=50 each) were released into the same Bugdorm cage in the morning (08:00-09:00) and left for an hour to disperse and establish territory. Wild-type females (n=50) were then added to the cages. Mating pairs were captured in 30 ml universal tubes with airholes and removed from the cage. Pairs were checked every 10 minutes and mating duration recorded. For each mating pair, the genotype of the male was determined based on the DsRed2 fluorescence marker, and the Relative Sterility Index (RSI) calculated as the proportion of females paired with transgenic males relative to all mating events, as previously described (Cayol et al., 1999).

The experiment was performed in triplicate and statistical significance was assessed by correction-free chi-square testing of pooled data, after it was confirmed by chi-square testing that there was no significant variation between replicates, in terms of female mating preference ($\chi^2 = 0.1318$, d.f. = 2, $p = 0.93$). After genotyping, males were removed from the vials and larval diet added for the female. Twenty females from each mating category were retained for two days, and then at least ten were dissected to assess the presence of sperm in the female genital tract. OX5122M heterozygous males did not vary significantly in mating competitiveness, relative to wild-type males (RSI = 0.5, $\chi^2 = 1$, d.f. = 1, $p > 0.999$, n = 136, **Table 4.7**). This indicates that, in the absence of defects caused by insertion site, expression of Ccprot1-zsGreen does not strongly impact on the ability of the heterozygous males to compete with wild-type males for mating events. There was a small, statistically significant difference in the mean mating duration (12 min) for WT (114 min) and OX5122M het males (126 min) when assessed by two sample t-test ($t = -2.31$, d.f. = 122, $p = 0.022$). This was no longer significant when replicates were assessed independently, which would control for handling

differences between the three people conducting the experiment (Ryan Turkel, Thea Marubbi and Charilaos Megas). Both mean durations of mating were less than the average for mass-reared WT males reported previously: 135 minutes (Shelly and Kennelly, 2002). The significance of this is considered subsequently (**Section 4.3.3**). The other OX5122 lines (OX5122D, K and G) were not assessed for mating competitiveness, but their ability to transfer marked sperm was considered.

Table 4.7: Mating preference of WT females in OX5122M competition assay

Replicate	Transgenic	WT	Total	RSI	Unmated females
1	25 (51%)	24 (49%)	49	0.51	1 (2%)
2	22 (48%)	24 (52%)	46	0.48	4 (8%)
3	21 (51%)	20 (49%)	41	0.51	9 (18%)
Summary	*68 (50%)*	*68 (50%)*	*136*	*0.50*	*14 (9%)*

The rate of mating refusal may have increased across replicates because tests were conducted 20-30 minutes apart; the peak rate of Medfly mating is in the early morning and late afternoon.

To assess the possibility of insertion site dependent variation in phenotype, three additional OX5122 lines (OX5122D, G & K) were assessed for transfer of fluorescently marked sperm (**Table 4.8**). Two groups were collected on eclosion and reared independently in small cages (n=25 per cage): wild-type females and OX5122 heterozygous males (all reared off-tet). As this test was not an assessment of mating competitiveness, WT males were not used. Wild-type females (n=25) were then added to the cages. A separate cage was used for each male genotype (OX5122D, G & K). Mating pairs were captured and confirmed to mate for at least one hour, prior to dissection. Control images (WT females mated to WT males) were shared with the previous experiment (OX5122M mating competitiveness), which was conducted one week previously.

Table 4.8: Mating frequencies of WT females and males of OX5122D, K and G

Line	Mated	Unmated	Total
OX5122D	22 (88%)	3 (12%)	25
OX5122K	22 (88%)	3 (12%)	25
OX5122G	21 (84%)	4 (16%)	25
WT	Images from the OX5122M competition assay were used		

In terms of sperm transfer (assessed one to two days after mating), all females mated to OX5122 heterozygotes (OX5122D, G, K & M) had brightly marked green

fluorescent sperm in the spermathecae (**Figure 4.7**). The quantity, morphology and motility of sperm was not obviously different for females mated to OX5122 males relative to WT, although an exact comparison would require PCR analysis, quantification of fluorescence, or counting of serial dilutions of spermathecal extracts under high magnification. These results indicated that the Ccprot1-zsGreen sperm marking system was a strong candidate for future commercial use, as it performed well in all four insertions tested. However, it would be interesting to develop a more quantitative assay to detect sperm transfer, for future use.

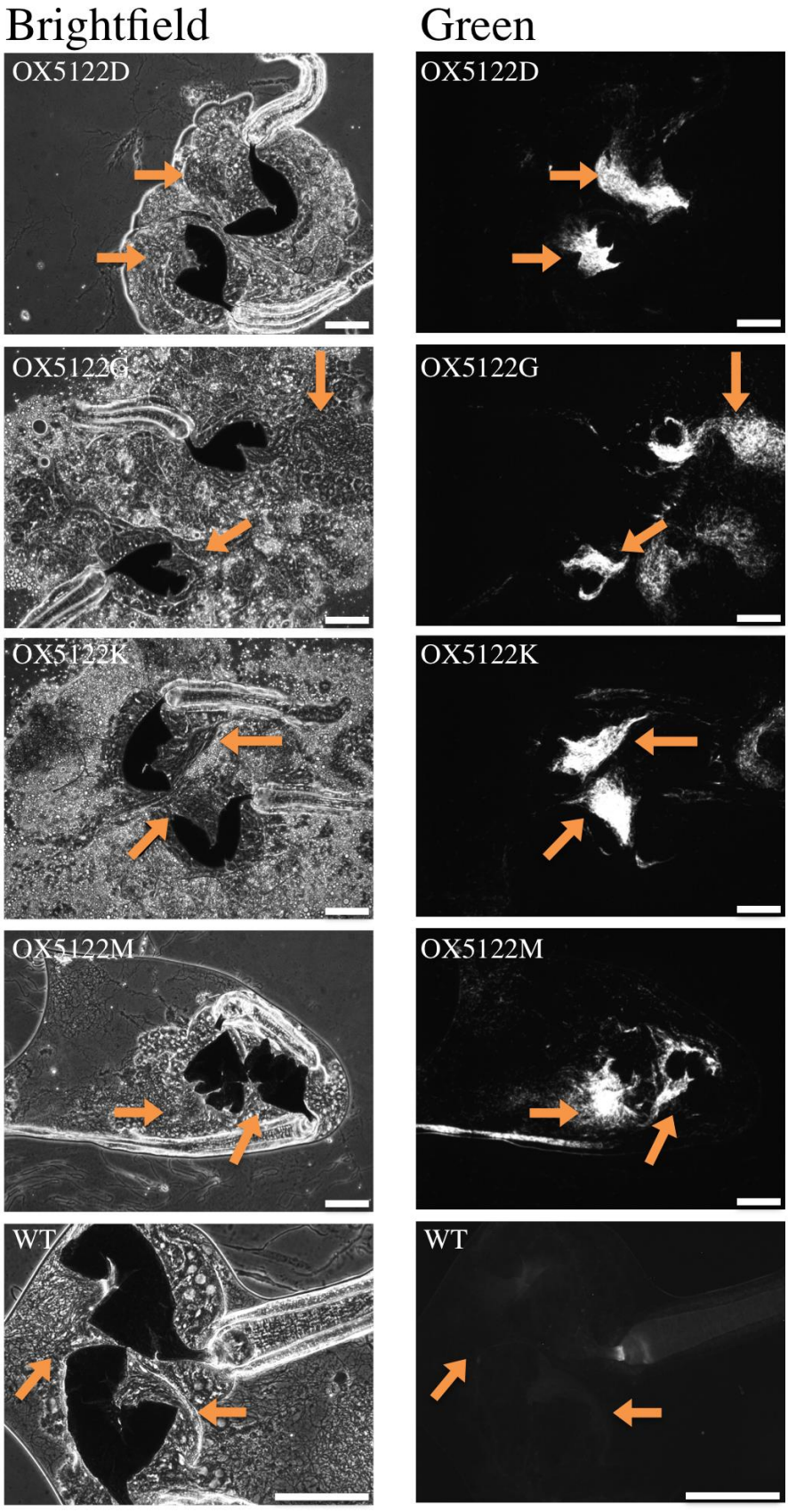


Figure 4.7. Heterozygous males of four OX5122 lines (OX5122D, G, K & M) transfer brightly marked sperm to females, with no evident difference in quantity or quality for any lines, relative to WT. Fluorescence and phase contrast microscopy of dissected spermathecae from WT females mated to WT or OX5122 males. The consistent transfer of fluorescently marked sperm (block arrows) across all four lines indicates that the marker system is minimally insertion sensitive. Scale bars: 100 μm.

4.3.2 Sperm of Ccprot1-zsGreen heterozygotes (OX5122M) remain visible after females are re-mated to WT males

It was previously shown that it was simple to differentiate between WT females mated once, to either OX5122M heterozygous males or WT males (**Section 4.3.1**), a key requirement for field application of the fluorescent sperm marking system. However, it had not yet been demonstrated that it was possible to identify WT females that had mated both a WT and OX5122 male. Species such as Medfly frequently accept second mates in the wild (Bonizzoni et al., 2002), (Kraaijeveld et al., 2005), which would reduce the suppressive ability of sterile male release, if females frequently re-mated with fertile wild males. This effect is magnified by a last mate precedence of paternity: more than 50% of offspring are sired from the male mated most recently, though this precedence decreases with time (Bertin et al., 2010), (Scolari et al., 2014). Because males sterilised with irradiation are generally less competitive and less able to induce refractoriness to re-mating than their non-sterile counterparts (Hendrichs et al., 2002), (Robinson et al., 2002), (Kraaijeveld and Chapman, 2004), it would be useful to investigate if the system for repressible male sterility developed in this study was associated with similar deficits.

We investigated the females' receptivity to mating with males of the opposite genotype to which they had already mated. The experiment would also indicate whether the transgene affected the males' ability or motivation to remate, though a full investigation of the effect of fluorescent sperm marker expression on re-mating tendency was outside the scope of this preliminary investigation. The analysis was performed with pairs captured in the prior mating competition experiment (OX5122M heterozygous: **Section 4.3.1**). We allowed the following four groups to recover overnight in small cages with fresh food and water, with 30 flies per cage: (1) wild-type males, (2) OX5122M heterozygous males, (3) wild-type females which had mated to wild-type males; and (4) wild-type females which had mated to OX5122M heterozygous males. The following morning, we set up two Bugdorm cages to facilitate re-mating with the opposite male genotype (**Table 4.9**). This experiment was similar to the mating competition study, with two exceptions: (1) males of only one genotype were used per group and (2) thirty males and thirty females were used per group. As before, males were allowed 1 hour to disperse prior to addition of females. Mating pairs were captured in 30 ml tubes, and the mating duration recorded. Males were removed, larval diet added to the tube and the female genital tract dissected one day later.

Females from the single mating experiment (**Section 4.3.1**) and this re-mating experiment, were dissected at the same time: at Cardiff University, as described previously.

Table 4.9: Experimental groups of the OX5122M/WT re-mating study

Group	Male	Female	
	Genotype	**Genotype**	**Partner for first mating**
1	WT	WT	OX5122M
2	OX5122M	WT	WT

We were not attempting to robustly assess variances in re-mating tendency, but it was obvious that a substantial minority (about 23%) of the females chose to re-mate; this did not appear to vary with the genotype of the first mating partner (**Table 4.10**). We were able to collect 8 females mated to OX5122M then WT males; and 6 females of the reciprocal group. One female from each group died prior to dissection; these were discarded from the analysis. These results gave an indication, though preliminary, that females mated to OX5122M males were not more likely to seek additional mates than females first mated to WT males. This was promising, because males of a commercially ideal transgenic line would induce female refractoriness to further re-mating, as efficiently as WT males do. However, a robust analysis to confirm this observation would require a larger sample size with replication, under conditions more similar to the field (larger cages with vegetation and reduced population density).

Table 4.10: Re-mating tendency of females previously mated to OX5122M or WT

Group	First partner	Second partner	Mated	Unmated
1	OX5122M	WT	8	22 (73%)
2	WT	OX5122M	6	24 (80%)

Fluorescent (OX5122M) and non-fluorescent (WT) sperm were observed in all but one female, independent of the genotype of the last mating partner. This single female had mated OX5122M prior to mating WT; it is possible that the first mating was associated with little or no transfer of sperm. This has been observed in other studies, though at a relatively infrequent proportion of total mating events (Taylor and Yuval, 1999), (Taylor et al., 2000), (Bertin et al., 2010). Due to the difficulty of simultaneously scoring fluorescence of hundreds of non-fluorescent and fluorescent sperm in a tightly overlapping space, it was not possible to make precise conclusions of the ratio of

unmarked to marked sperm. On average, it appeared that females mated to OX5122M then WT demonstrated a lesser intensity of fluorescent sperm marking than females mated singly to OX5122M, or to WT then OX5122M (**Figure 4.8**).

Initially, this was taken to indicate that transgenic sperm were successfully able to displace wild-type sperm of a prior male. However, this conclusion was contradicted by several observations. First, the number of sperm transferred to the spermathecae after a mating event is highly variable. In three studies with laboratory reared strains, the mean number of sperm transferred to the spermathecae was determined as 3192 (range: 140-10400) in the first, 3576 (range not provided) in the second, and 1173 (range: 0-4300) in the third (Taylor and Yuval, 1999), (Twig and Yuval, 2005), (Bertin et al., 2010). In a study with wild-caught males, the median number of sperm transferred was 2608 (range: 285-9249) (Taylor et al., 2000). Therefore, it is highly likely that the observed variations in the intensity of fluorescence, resulted from differences in the number of sperm transferred, rather than direct displacement. There were additional factors that suggested that direct displacement did not occur. First, experimenter bias was possible, as the genotypes of all individuals were known. To address this, we applied double blind scoring in subsequent experiments. Secondly, the storage of sperm in the spermathecae is generally asymmetric (Taylor and Yuval, 1999), (Twig and Yuval, 2005), which may have led to an overestimation of the proportion of fluorescently marked sperm, relative to conditions where the distribution of sperm was more equal. This was likely, because it was not possible to visually differentiate all unique sperm within tightly aggregated clusters, using the microscope conditions applied in the study. Finally, the number of sperm transferred to the spermathecae from the first and second partner has been shown to be approximately equal (Bertin et al., 2010), which indicates that active displacement in the spermathecae does not occur, at least not in a sense equivalent to that suggested for *D. melanogaster* (Price et al., 1999).

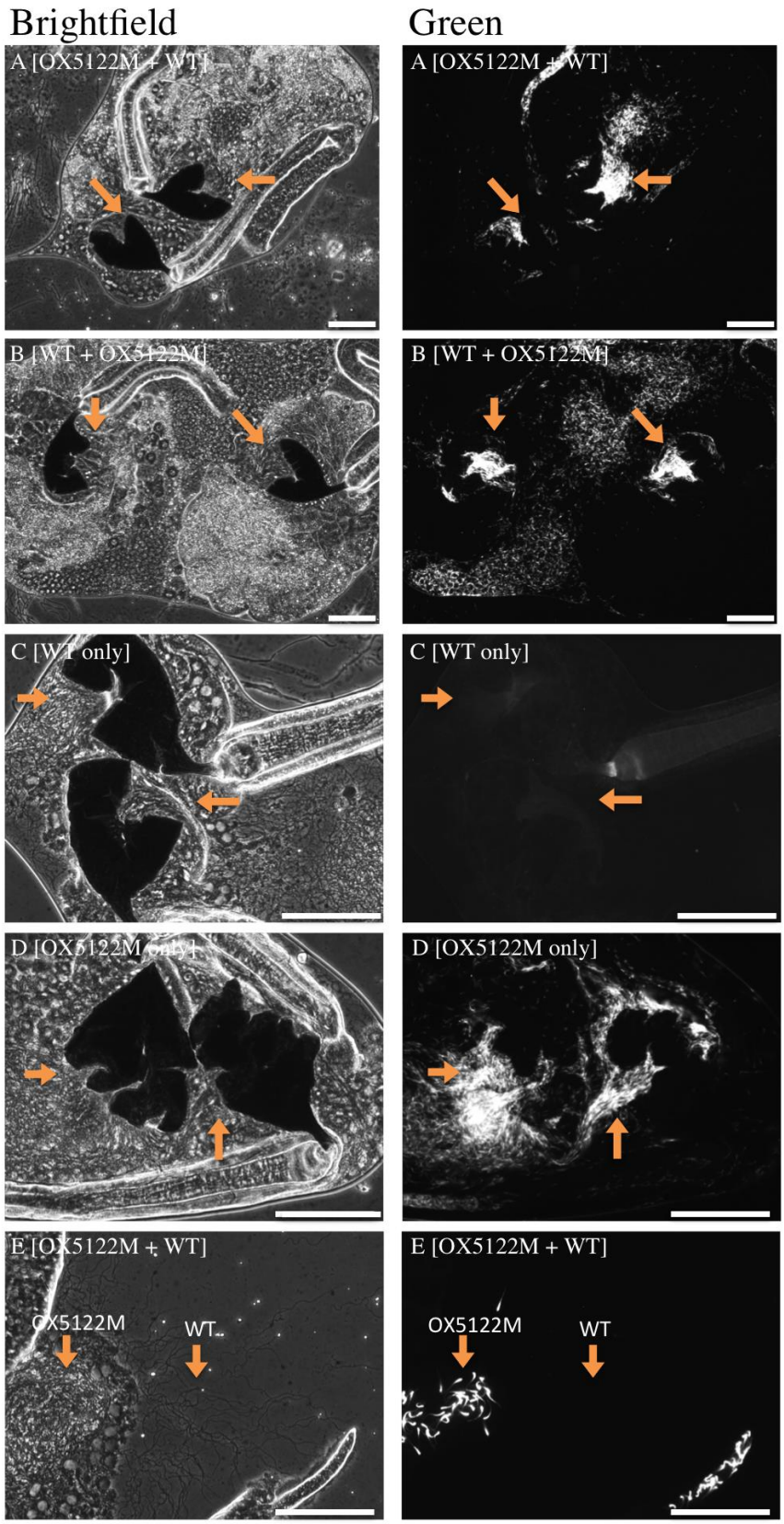


Figure 4.8. OX5122M heterozygous males transfer fluorescently marked sperm that persist within the spermatechae after re-mating to WT males. Fluorescence and phase contrast microscopy of dissected spermathecae from WT females mated to multiple males: OX5122M then WT (**A**) or WT then OX5122M (**B**). Sperm indicated with block arrows. Single matings to WT (**C**) or OX5122M (**D**) shown for reference. On average, it appeared that females mated to OX5122M then WT appeared to demonstrate a lesser intensity of fluorescent sperm marking than females mated singly to OX5122M, or to WT then OX5122M. (**E**) WT (unmarked) and OX5122M sperm (marked) in a twice mated female. Scale bars: 100 μm.

Instead, the last mate precedence in paternity is likely to be mediated by stratification of sperm in the fertilisation chamber. Fluorescence microscopy of the fertilisation chamber of females mated twice, to males with differentially marked fluorescent sperm, has been investigated by another group. This indicated a greater concentration of sperm from the second partner near the central portion of the alveoli, which could bias paternity to the second partner (Marchini et al., 2001), (Scolari et al., 2014). Two further pieces of information are relevant. First, the quantity of sperm in the fertilisation chamber reaches a peak immediately after mating, which declines in the subsequent days (Twig and Yuval, 2005). Secondly, it was demonstrated that males partition the transfer of sperm between the storage organs of females, with about 20% of sperm directed to the fertilisation chamber and 80% to the spermathecae (Bertin et al., 2010). Therefore, the most parsimonious explanation of the last mate precedence in paternity is that a comparatively greater quantity of sperm from the most recent mate, is localised in the areas of the fertilisation chamber through which eggs pass prior to oviposition (Marchini et al., 2001), (Scolari et al., 2014). Thereafter, these sperm are depleted through fertilisation and the chamber is replenished by the spermathecae, which appears to retain sperm of the first and second males, in the same proportion as these males originally transferred. In other words, there is a stratification of sperm from the most recent mate in the fertilisation chamber, but not the spermathecae.

It would be interesting to repeat this study with double blind scoring, to confirm beyond reasonable doubt that an experienced person could differentiate females that had mated a single WT male, a single OX5122 male, or twice (to both male genotypes). We decided to wait until an adequate system for repressible male sterility had been developed, as it was likely that expression of this effector could influence female decision to remate. Therefore, it would have been unwise to devote excessive study to the dynamics of remating, at this stage.

4.3.3 Ccprot1-zsGreen homozygotes (OX5122M-hom1) are competitive for mates and transfer healthy marked sperm to females

As product lines are maintained as homozygous colonies, we next evaluated if homozygosity would enhance fluorescence intensity, reduce male mating competitiveness, or affect the dynamics of sperm transfer. To ensure comparability with the heterozygous situation we again selected the single autosomal insertion line OX5122M, which was previously assessed for mating competitiveness and the dynamics of sperm transfer (**Sections 4.3.1-4.3.2**). The line was made homozygous by random single pair crosses, as previously described (**Figure 2.5**). This quickly establishes homozygosity, but potentially reduces fitness by constraining genetic diversity to two individuals. First, the transgenic allele was enriched by crossing transgenics for two generations. Thereafter, twenty single pair crosses were set up to establish homozygous pools. Twelve of twenty pools (60%) were productive (three refused mating; five did not yield sufficient progeny to continue). Then, G_3 progeny were screened to eliminate crosses of two heterozygotes (indicated by WT progeny). Six of twelve pools were eliminated, leaving pools where one or both parents were homozygous. Finally, homozygous pools were discriminated from pools with one homozygous and one heterozygous parent. Siblings from the six remaining pools were crossed in medium cages for two more generations to facilitate selection of truly homozygous pools (no WT progeny). Two homozygous pools were obtained.

One homozygous pool was randomly selected (OX5122M-hom1) and assessed for mating competitiveness, as described for the heterozygous OX5122M line (**Section 4.3.1**); tests were conducted one hour later, between 10:00-11:00. The data across three replicates (**Table 4.11**) were pooled after verifying that there were no significant differences between replicates in terms of female mating preference ($\chi^2 = 0.313$, d.f. = 2, $p = 0.855$). We found no significant difference in competitiveness between transgenic males and WT males (RSI = 0.49, $\chi^2 = 0.008$, d.f. = 1, $p = 0.927$, n = 116). There was no significant difference in mean mating duration of OX5122M-hom1 males (142 min) relative to WT (138 min) when assessed by independent two-sample t-test (two-tailed, $t = -0.47$, d.f. = 91, $p = 0.638$). It is interesting to note that in this instance, the mean mating duration for both groups were greater than those reported in published study: 135 minutes (Shelly and Kennelly, 2002). This was the third time the study had been performed: for the OX4718A-resolved assay (**Section 3.6**), the mean mating durations were 106 min (WT) and 114 min (OX4718A-resolved). For the OX5122M-het assay

(**Section 4.3.1**), the mean mating durations were 114 min (WT) and 126 min. The gradual increase in the observed durations of mating, suggests that experience with the assay is a possible explanatory factor.

Table 4.11 Mating preference of WT females in OX5122M-hom1 competition assay

Replicate	Transgenic	WT	Total	RSI	Unmated
1	18 (46%)	21 (54%)	39	0.46	11 (22%)
2	23 (52%)	21 (48%)	44	0.52	6 (12%)
3	18 (50%)	18 (50%)	36	0.50	14 (28%)
Summary	*59 (50%)*	*60 (50%)*	*119*	*0.50*	*31 (21%)*

The number of females refusing to mate in this study (21%) may have been greater than in the previous study of OX5122M heterozygotes (13%, **Section 4.3.1**) as it was conducted one hour later. Future studies were designed to start at the same time (09:00), to control for this potentially important variable.

We also considered the possibility of reduced sperm motility or fluorescence intensity after mating to WT females (ie. after sperm passed into the female reproductive tract). Females were processed, generally as described previously (the male was removed; larval diet was supplied; the female transferred to Cardiff University and dissected one, rather than two, days later). The motility and quantity of sperm transferred to females, did not appear to differ between OX5122M-hom1 males and WT males. When comparing images of females mated to OX5122M-hom1 males (this study) and OX5122M-het males (**Section 4.3.1**), we did not notice an obvious difference in fluorescence intensity (**Figure 4.9**). However, it would be necessary to compare images of the reproductive tract of females mated to OX5122M heterozygous males and homozygous males obtained at the same time, with equivalent camera settings, if this was necessary to completely exclude this possibility. This was not pursued, because the system clearly performed adequately for future use in product development.

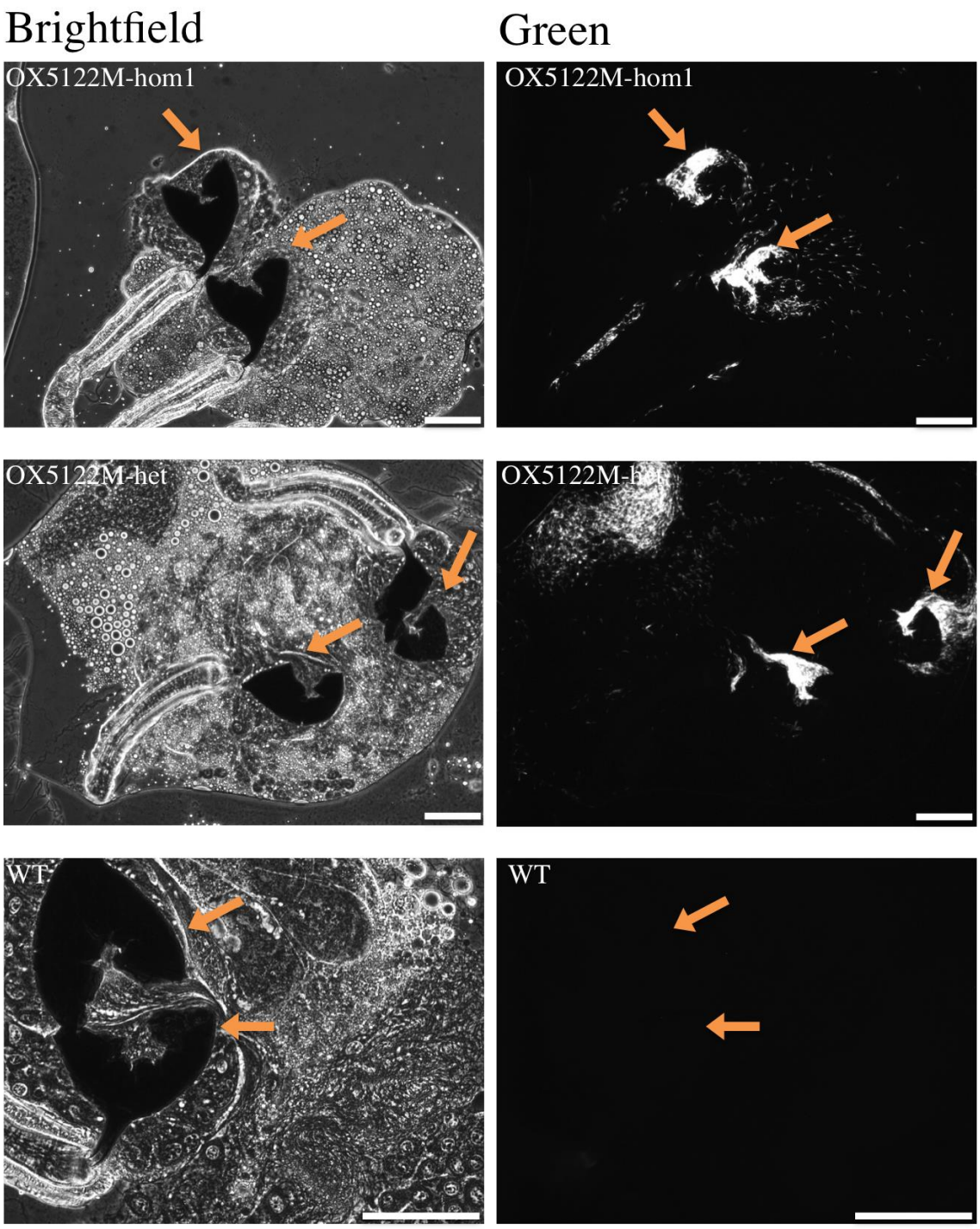


Figure 4.9. OX5122M homozygous males transfer bright, fluorescently marked sperm to females, with no evident difference in quantity or quality, relative to WT or OX5122M heterozygous males. Fluorescence and phase contrast microscopy of dissected spermathecae from WT females mated to WT, OX5122M heterozygous or OX5122M homozygous males. Sperm indicated with block arrows. We did not detect an obvious difference in the intensity of fluorescent marker expression between OX5122M heterozygotes and homozygotes. Scale bars: 100 μm

4.3.4 The fluorescence of Ccprot1-zsGreen (OX5122M-hom1) sperm is highly persistent in dead females trapped under field-like conditions

To evaluate the field relevance of the fluorescent sperm marking system, a marker persistence assay simulating wild conditions was devised. WT females of two groups (mated to WT males or OX5122M-hom1 males) were affixed to a yellow sticky trap for 0, 1, 7, or 14 days. We assessed 10 females of each group, for each timepoint (80 total). Spermathecae were dissected in PBS, prior to gentle squashing under a cover slip and microscopic observation at Oxitec (Motic BA210 microscope and Fraen fluorescence FLUOLED lamp, at 10x magnification). The male genotype to which females were mated was genotyped by the presence or absence of fluorescently marked sperm (OX5122M-hom1 or WT, respectively). Double blind scoring was applied, to preclude experimenter bias. The male was correctly identified in most instances (72/79; 91%), even after the female was left on a trap for 2 weeks **(Table 4.12**). This was the first time the assessment was performed; it is possible that with practice the accuracy would be increased.

Table 4.12 Field simulation of Ccprot1-zsGreen (OX5122M-hom1) sperm marking persistence in mated females

Days on trap	**Accuracy of scoring**		
	Correct	**Incorrect**	
		WT misidentified as OX5122	**OX5122M misidentified as WT**
0	17/20	1/20	2/20
3	17/19	1/19	1/19
7	19/20	0/20	1/20
14	19/20	0/20	1/20

One sample was lost during processing in the day 3 group.

4.4 Modification of Ccprot1 and Ccprot2 to regulate effectors of repressible male sterility

4.4.1 Identification of the Ccprot1 and Ccprot2 transcriptional start sites

Having developed functional expression systems for a fluorescent marker in the male germline (Ccprot1-zsGreen [OX5122]; Ccprot2-zsGreen [OX5150]), we next attempted to modify these components to regulate a repressible male sterility effector, for future application in a single construct incorporating both components. From prior results (**Section 4.2**), we concluded that both Ccprot1-zsGreen (OX5122) and Ccprot2-

zsGreen (OX5150) functioned equivalently when integrated at a favourable genomic insertion site. It was possible that Ccprot1-zsGreen (OX5122) was less insertion sensitive than Ccprot2-zsGreen (OX5150), because we observed impenetrant OX5150 phenotypes more frequently. Consequently, we decided to first develop a construct based on Ccprot1, and revert to Ccprot2 if the former was non-functional. The changes required to mediate repressible male sterility are summarised here.

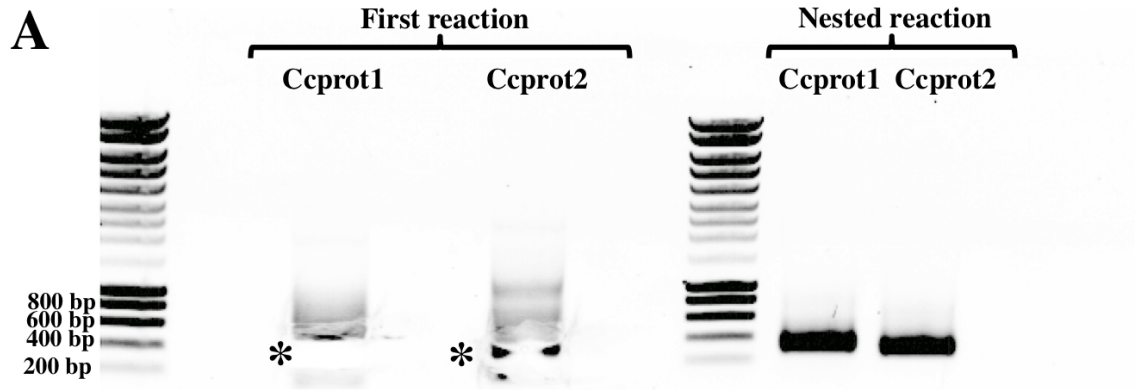
First, substitution of FokI for zsGreen was required, to replace fluorescent reporter localisation with protamine-FokI mediated cleavage of the sperm genome. Thereafter, integration with the previously described, tetracycline-repressible male germline switch (tetO21-β2T[short]-Cchsp83-tTAV) was necessary. This involved placement of a Ccprot-FokI system in a head-to-head configuration to the male germline switch, to put the tetO21 site upstream of the Ccprot-Fok1 transcription unit, and thus facilitate tetracycline repression. Finally, substitution of the Ccprot1 promoter for a minimal promoter fragment (from Dmhsp70) was required. Minimal promoters are generally required for tetracycline responsive systems, where they facilitate tight repression, with minimal transgene expression when tetracycline is present (Stebbins and Yin, 2001), (Schönig et al., 2011). The Ccprot1 promoter fragment is substantially longer (1.4 kb) than Dmhsp70 minimal promoter, and considering the intensity of reporter translation in the male germline, evidently quite strong (**Figure 4.3**). Therefore, it was possible that its inclusion in a tet-repressible system, might reduce sensitivity to tetracycline. Considering that tetO-Dmhsp70 minimal promoter-protamine-FokI fragments were previously demonstrated (in OX4353/4718A/5036; **Chapter 3**) to mediate penetrant and repressible male sterility, the argument for replacing the Ccprot1 promoter with this fragment was compelling.

To facilitate ligation of tetO-Dmhsp70 minimal promoter to Ccprot1 or Ccprot2 5'UTR without disrupting the regulatory capacity of each component, we wished to identify the transcriptional start site (TSS) by 5' RACE. For convenience, RNA that had been extracted from the phenotypically normal testes of OX5140E (Dmprot1-zsGreen) flies, rather than a fresh extraction from WT testes, was used for the 5' RACE. Nested PCR reactions were performed with gene-specific reverse primers for Ccprot1 and Ccprot2; and the proprietary 5'RACE forward primer mix (UFP1 and UFP2). The position of each gene-specific reverse primer relative to the first codon (ATG) is provided (**Table 4.13**). After confirming the presence of a specific product in the second

(nested) reaction, the dominant band of the first reaction PCR product (pre-nesting) was gel purified. Thereafter, it was cloned into the pJET vector and ten individual colonies were sequenced with the protamine-specific reverse primer of the second (nested) PCR reaction. All ten Ccprot1 clones generated high quality sequence, while only 7/10 Ccprot2 clones gave good sequence (potentially an issue with the gene-specific reverse primer used for sequencing). The results did not indicate a single TSS for either Ccprot1 or Ccprot2. We investigated the Medfly RNA-seq data on the NCBI database to compare our results to those obtained via high throughput transcript sequencing (**Figure 4.10**). Between the 5' RACE result and the high-throughput RNA sequencing data, we chose to use the most upstream of the potential TSS, to enhance the probability of retaining the ability of the Ccprot1 or Ccprot2 5'UTR to mediate translational repression.

Table 4.13 Positions of gene-specific 5' RACE primers, relative to first codon

Name	Target	Nested PCR reaction	Position relative to ATG (+1)
TD3938	Ccprot1	First	+ 188 to 209
TD3939		Second	+ 92 to 110
TD3940	Ccprot2	First	+ 101 to 122
TD3941		Second	+ 72 to 92



B

Ccprot1	**[49 bp]…AATCGTACAAATTCTAGTGAAAACGATATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG**
#10	AATCGTACAAATTCTAGTGAAAACGATATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#8	AAATTCTAGTGAAAACGATATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#2,3	ATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#1,9	TACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#11	ATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#4,7	AGTTATAAAAAATTTGTTG…[106 bp]…ATG
#5	G…[106 bp]…ATG
Ccprot2	**[25 bp]…AGACAATTGAAAATTTCCAGTCAT…[114 bp]…ATG**
#3	AGACAATTGAAAATTTCCAGTCAT…[114 bp]…ATG
#6,7	GAAAATTTCCAGTCAT…[114 bp]…ATG
#1,8	AT…[114 bp]…ATG
#2,4,5	T…[114 bp]…ATG

C

Ccprot1 (Annotation)	**TGTATATCATTTTAAAACCATTTGCGCAAATACATTTGAAAATGTTTTCA…[157 bp]…ATG**
Ccprot1 (RNA-seq)	TATCATTTTAAAACCATTTGCGCAAATACATTTGAAAATGTTTTCA…[157 bp]…ATG
Ccprot1 (5' RACE)	A…[157 bp]…ATG
Ccprot2 (Annotation)	**…[26 bp]…AGACAATTGAAAATTTCCAGTCATTCGTTTTACATTTTTTTT…**[97 bp]…ATG
Ccprot2 (RACE)	AGACAATTGAAAATTTCCAGTCATTCGTTTTACATTTTTTTT…[97 bp]…ATG
Ccprot2 (RNA-seq)	ATTTTTTTT…[97 bp]…ATG

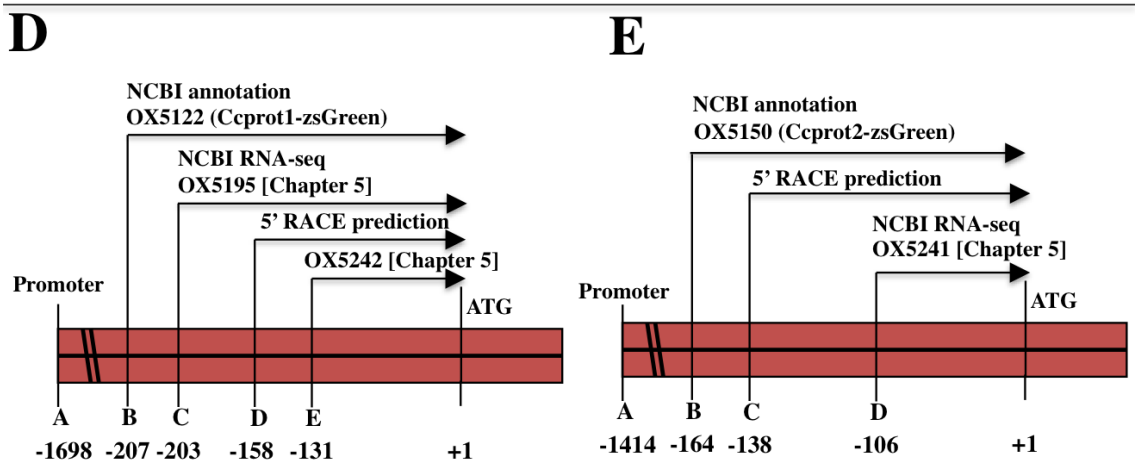


Figure 4.10. Investigation of the TSS in Ccprot1 and Ccprot2 to design an effector for repressible male sterility. (A-B) 5' RACE on Ccprot1 and Ccprot2. (**A**) Nested PCR reactions to isolate 5' RACE product (asterisks show extracted portion of gel). Expected sizes: CcProt1 [#1] (416 bp), CcProt1 [#2] (317 bp), CcProt2 [#1] (286 bp), CcProt2 [#2] (256 bp). Ten clones of the dominant band of the pre-nested reaction (*) were sequenced. (**B**) Seven unique sites were indicated for Ccprot1 and four for Ccprot2. In both cases, the TSS indicated by 5' RACE was downstream of the annotation from NCBI (Ccprot1: 49 bp, Ccprot2: 25 bp). (**C**) Alignment of potential TSS for Ccprot1 and Ccprot2: annotation from NCBI; NCBI RNA-seq data (furthest upstream sequence); and 5' RACE (furthest upstream sequence). (**D-E**) Diagrams of sequences on Ccprot1 (**D**) and Ccprot2 (**E**). Annotations: upstream terminus of the protamine promoter; predictions of the TSS (NCBI annotation, 5'RACE and NCBI RNA seq); versions applied in future constructs (**Chapter 5**) as tetO-Dmhsp70-Ccprot chimerae for repressible male sterility.

4.4.2 Dmhsp70 promoter-Ccprot1 5'UTR-zsGreen fusions (OX5182, OX5184 & OX5186) are translationally repressed in the male germline

We next tested whether transcripts expressed from a construct containing a fusion of Dmhsp70 promoter (not Dmhsp70 **minimal** promoter) to Ccprot1, in a similar conformation to those applied for effectors of repressible male sterility, would retain the translational profile conferred by the Ccprot1 5'UTR (delayed to elongating spermatid stage: **Figure 4.3**). The full-length Dmhsp70 promoter was used here, because the minimal promoter was not expected to mediate high levels of transcription, if not activated by tTAV at its target tetO sequences. We tested three fusions of variable lengths of the Dmhsp70 promoter fragment to Ccprot1 5'UTR-Ccprot1 ORF-**zsGreen**-Ccprot1 3'UTR, to observe localisation of zsGreen in the male germline (**Figure 4.11**). All promoter fragments retained less of the predicted Dmhsp70 5'UTR, relative to previous constructs (OX4353/4718/5036: 89 bp). The lengths of predicted Dmhsp70 5'UTR retained were: 16 bp (OX5182), 21 bp (OX5184), or 31 bp (OX5186). Previous constructs using the Dmhsp70 minipromoter and 5'UTR had generated transcripts that were not subject to translational delay. Therefore, we attempted to remove the majority of Dmhsp70 5'UTR from the fragment whilst retaining its promoter capacity; and to delay translation by inclusion of the Ccprot1 5'UTR. This could potentially remove the negative effects on sperm fitness observed in the prior system (OX4718, **Figure 3.8**).

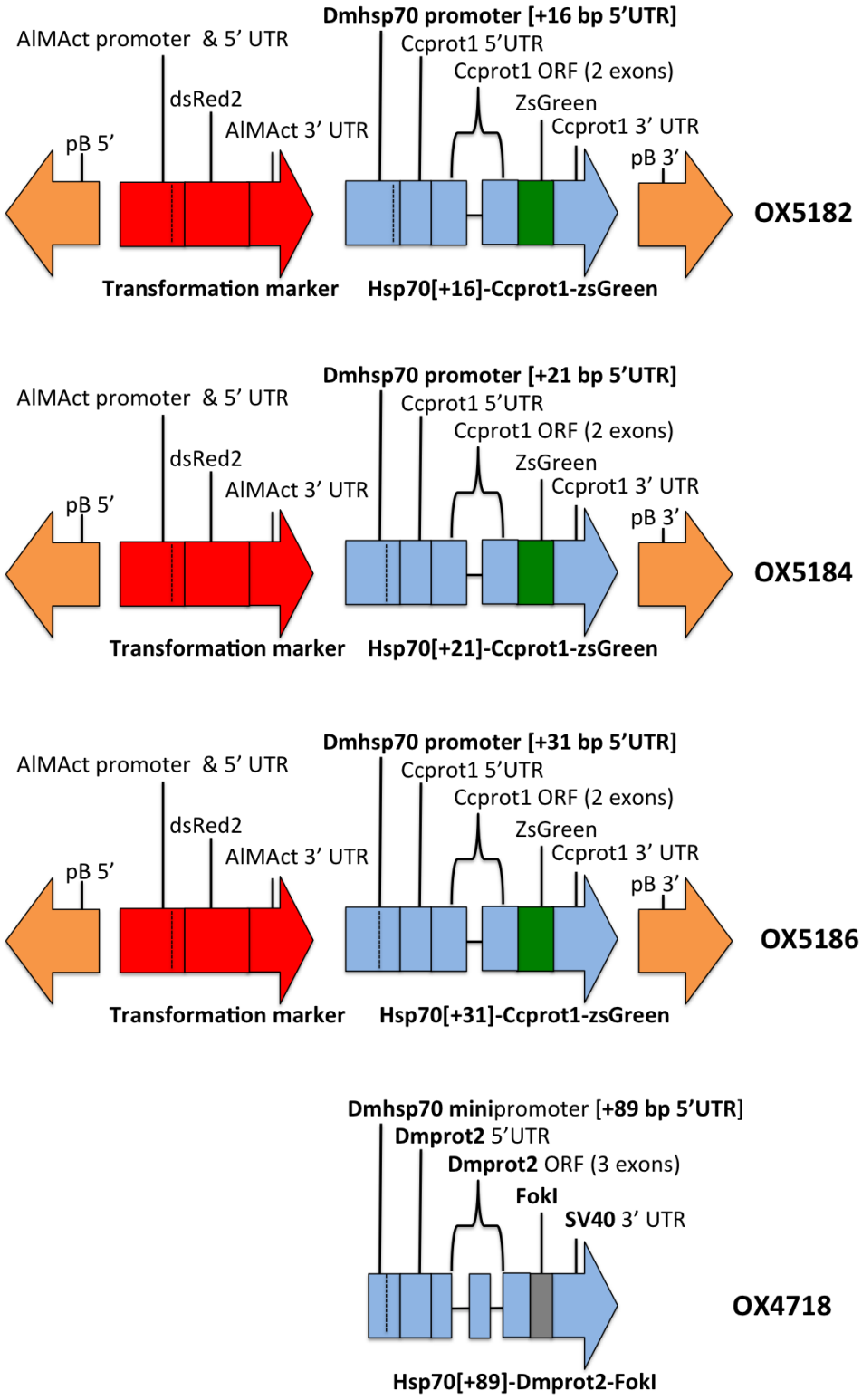


Figure 4.11. DmHsp70 promoter-Ccprot1-zsGreen reporter fusion constructs (OX5182, OX5184 & OX5186) to test their suitability for the development of male sterility effectors. Variable lengths of the Dmhsp70 promoter fragment (incorporating a predicted length of 16, 21 or 31 bp of Dmhsp70 5'UTR) were fused to Ccprot1 5'UTR-Ccprot1 ORF-**zsGreen**-Ccprot1 3'UTR. Dashes indicate promoter-5'UTR boundary. All constructs featured the previous transformation marker (AlMAct-dsRed2) and unused sites for site-specific recombination (not shown). These sites were artefacts of the prior vector: upstream of AlMact-dsRed2 (loxp, frt3, frt, attp58) and downstream of the protamine-reporter fragment (attp220). The prior fragment from OX4718 (incorporating a Dmhsp70 minipromoter fragment, rather than full-length promoter, and including a predicted 89 bp of hsp70 5'UTR), is shown for reference.

Microinjection (**Table 4.14**) and backcrossing to WT (**Table 4.15**) were performed with *piggyBac* helper OX3022 (300 ng/µl) and one of three expression constructs OX5182, OX5184, or OX5186 (600 ng/µl). To verify successful microinjection, surviving G_0 pupae were screened for transient expression of the AlMAct-dsRed2 transformation marker: this was observed for all constructs. Adult survival was better than the average rate for Medfly microinjections at Oxitec (25%), for OX5182 (53%) and OX5184 (56%); and less than average for OX5186 (14%) (Gregory et al., 2016). Six lines were kept from each construct for phenotypic analysis. From each pool, one transgenic G_1 individual was backcrossed to wildtype and eggs collected six days later to establish a transgenic line. As previously described, progeny of each transgenic line were assessed for Mendelian characteristics: insertion copy number and sex-linkage (**Table 4.16**).

Table 4.14: Microinjection logistics for OX5182, OX5184, & OX5186

Construct	Embryos	Larvae	Pupae	Adults	Lines
OX5182	450	348 (77%)	244 (54%)	240 (53%)	10 (4%)
OX5184	444	377 (85%)	292 (66%)	248 (56%)	10 (4%)
OX5186	550	134 (24%)	90 (16%)	75 (14%)	7 (9%)

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (transgenic lines/G_0 adults crossed). Although the apparent transformation efficiency is lower for OX5182 and OX5184 than for OX5186, this appears to be an artifact of pool size (smaller pools were used in OX5186).

Table 4.15 G_0 backcrosses to establish OX5182, OX5184 & OX5186 lines

OX5182A	**20 ♂ x 20 WT ♀**	**OX5182B**	**20 ♂ x 20 WT ♀**	**OX5182C**	**20 ♂ x 20 WT ♀**
OX5182D	20 ♂ x 20 WT ♀	**OX5182E**	**20 ♂ x 20 WT ♀**	**OX5182F**	**23 ♂ x 23 WT ♀**
OX5182G	20 ♀ x 10 WT ♂	**OX5182H**	**20 ♀ x 10 WT ♂**	**OX5182I**	**20 ♀ x 10 WT ♂**
OX5182J	**20 ♀ x 10 WT ♂**	**OX5182K**	**20 ♀ x 10 WT ♂**	**OX5182L**	**24 ♂ x 44 ♀**
OX5184A	**20 ♂ x 20 WT ♀**	**OX5184B**	**20 ♂ x 20 WT ♀**	**OX5184C**	**20 ♂ x 20 WT ♀**
OX5184D	20 ♂ x 20 WT ♀	**OX5184E**	**20 ♂ x 20 WT ♀**	**OX5184F**	**20 ♂ x 20 WT ♀**
OX5184G	**20 ♀ x 10 WT ♂**	**OX5184H**	**20 ♀ x 10 WT ♂**	**OX5184I**	**20 ♀ x 10 WT ♂**
OX5184J	20 ♀ x 10 WT ♂	**OX5184K**	**20 ♀ x 10 WT ♂**	**OX5184L**	**20 ♀ x 10 WT ♂**
OX5186A	5 ♀ x 5 WT ♂	OX5186B	5 ♀ x 5 WT ♂	OX5186C	5 ♀ x 5 WT ♂
OX5186D	5 ♀ x 5 WT ♂	OX5186E	5 ♂ x 20 WT ♀	**OX5186F**	**5 ♂ x 20 WT ♀**
OX5186G	**5 ♂ x 20 WT ♀**	OX5186H	5 ♂ x 20 WT ♀	OX5186I	5 ♂ x 20 WT ♀
OX5186J	**5 ♂ x 20 WT ♀**	**OX5186K**	**5 ♂ x 20 WT ♀**	**OX5186L**	**3 ♀ x 5 WT ♂**
OX5186M	**4 ♀ x 5 WT ♂**	**OX5186N**	**8 ♂ x 25 WT ♀**	OX5186O	5 ♀ x 5 WT ♂

Underlined pools yielded transgenics. OX5182L contained both male and female injection survivors. 22 adults (8 males and 14 females) of OX5184 injection survivors were not crossed. Assessed lines were **OX5182**B, C, E, F, I & J; **OX5184**A, B, C, E, F & K; and **OX5186**F, G, J, L, M & N.

Table 4.16 Mendelian analysis of OX5182, OX5184 & OX5186 transgenic lines

Line	**Sex**	**Copy number**		**Sex-linkage**		**Summary**		**Fluorescence in testes**
		Transgenic (%)	n	Sex ratio (M/F)	n	Copies	Location	
OX5182C	♂	49	169	1.1	64	1	Autosome	**No**
OX5182E	♂	54	149	1.0	74	1	Autosome	Yes
OX5182F	♂	**78**	116	1.5	54	**2**	Autosome	**No**
OX5182I	♂	**81**	90	2.0	42	**2**	Autosome	Yes
OX5182J	♂	47	110	0.9	50	1	Autosome	Yes
OX5182B	♀	51	89	1.8	33	1		**No**
OX5184A	♂	49	170	1.2	76	1	Autosome	Yes
OX5184C	♂	46	72	1.4	29	1	Autosome	Yes
OX5184E	♂	55	124	0.9	62	1	Autosome	Yes
OX5184F	♂	56	109	1.4	50	1	Autosome	Yes
OX5184B	♀	45	71	0.5	19	1		**No**
OX5184K	♀	62	68	0.7	19	1		Yes
OX5186F	♂	47	47	Male-only	13	1	Y	**No**
OX5186G	♀	48	109	1.2	40	1		**No**
OX5186J	♀	53	83	1.3	27	1		**No**
OX5186L	♀	46	90	0.9	28	1		Yes
OX5186M	♀	55	99	3.0	40	1		Yes
OX5186N	♀	43	68	1.8	25	1		Yes

All lines appear to be single insertions, except OX5182F and I (potential double insertions). Sex-linkage is only apparent from crosses in which the transgenic parent is male. Where it was possible to determine linkage, all lines appeared autosomal except OX5186F (Y-linkage). “N” refers to number screened.

Heterozygous G_2 OX5182, OX5184, or OX5186 males (n=10) were collected on eclosion in small cages. Males aged 5-7 days post-eclosion were dissected at Cardiff University, as previously described. Testes from at least five males per line were dissected in testis buffer, gently pressed under a coverslip and examined to identify zsGreen reporter localisation in spermatocytes, spermatids and sperm (**Figures 4.12-4.14)**. Approximately half of lines demonstrated no visible fluorescence in testes (7/18). In the other lines, expression was generally observed in spermatids and sperm, but not spermatocytes. This suggested pre-meiotic transcription mediated by the truncated Dmhsp70 promoter variants, with delayed translation mediated by Ccprot1 5'UTR, as desired for the appropriate expression of effectors for repressible male sterility. It is interesting to note that stochastic variation was observed in expression between some lines, and within cells of the same individual. For instance, spermatids are poorly marked in OX5182I, but not OX5182E. In OX5184F, spermatids of similar age were marked with varied intensity. This was not observed during the analysis of OX5122, which featured a full-length Ccprot1 promoter. Therefore, it was anticipated that the penetrance of male sterility would be relatively insertion sensitive in future tetO-Dmhsp70-Ccprot1-FokI lines.

Furthermore, the reporter was frequently localised to spermatids that had not completed elongation (for instance in OX5184E, **Figure 4.13**); localisation of protamine-FokI at this stage could still potentially affect spermatid nuclear shaping, and therefore reduce the rate of individualisation and the proportion of healthy mature sperm. The marker expression phenotypes observed between the three constructs were similar, indicating that all were fit-for-purpose in future research and development. However, it remained to be proven that this expression profile would be retained when combined with a tetracycline-repressible system, and that the sperm would be minimally affected by the expression of FokI.

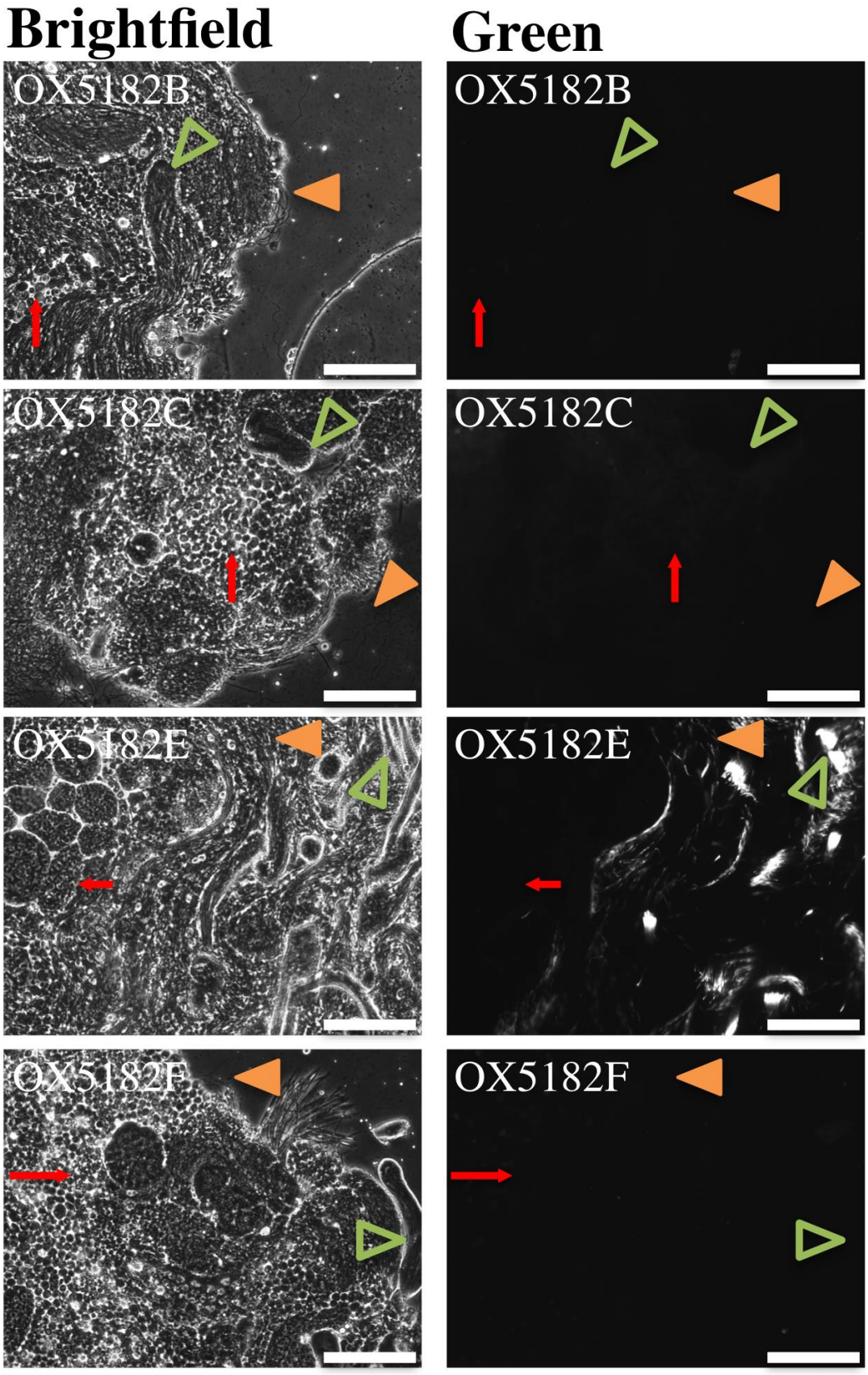


Figure 4.12. DmHsp70 promoter [+16 bp Dmhsp70 5'UTR]-Ccprot1-zsGreen (OX5182) mediates reporter expression in the male germline of three of six tested lines (OX5182E, I & J) [continued on the next page]. Fluorescence and phase contrast microscopy of Medfly testes demonstrates fluorescent reporter localisation in sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), of most lines with visible reporter expression. Scale bars: 100 µm.

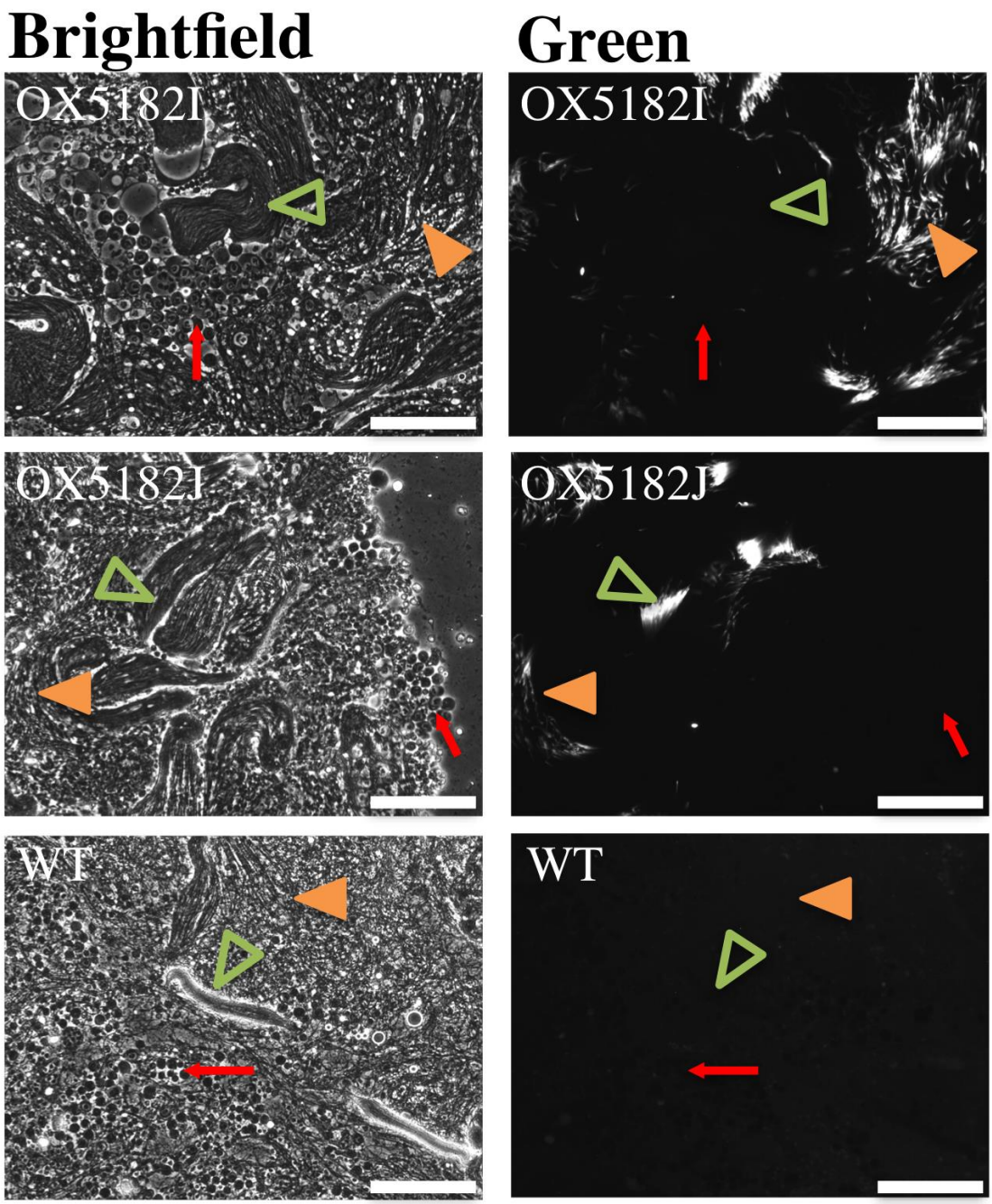


Figure 4.12. DmHsp70 promoter [+16 bp Dmhsp70 5'UTR]-Ccprot1-zsGreen (OX5182) mediates reporter expression in the male germline of three of six tested lines (OX5182E, I & J).
Fluorescence and phase contrast microscopy of Medfly testes demonstrates fluorescent reporter localisation in sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), of most lines with visible reporter expression. Scale bars: 100 μm.

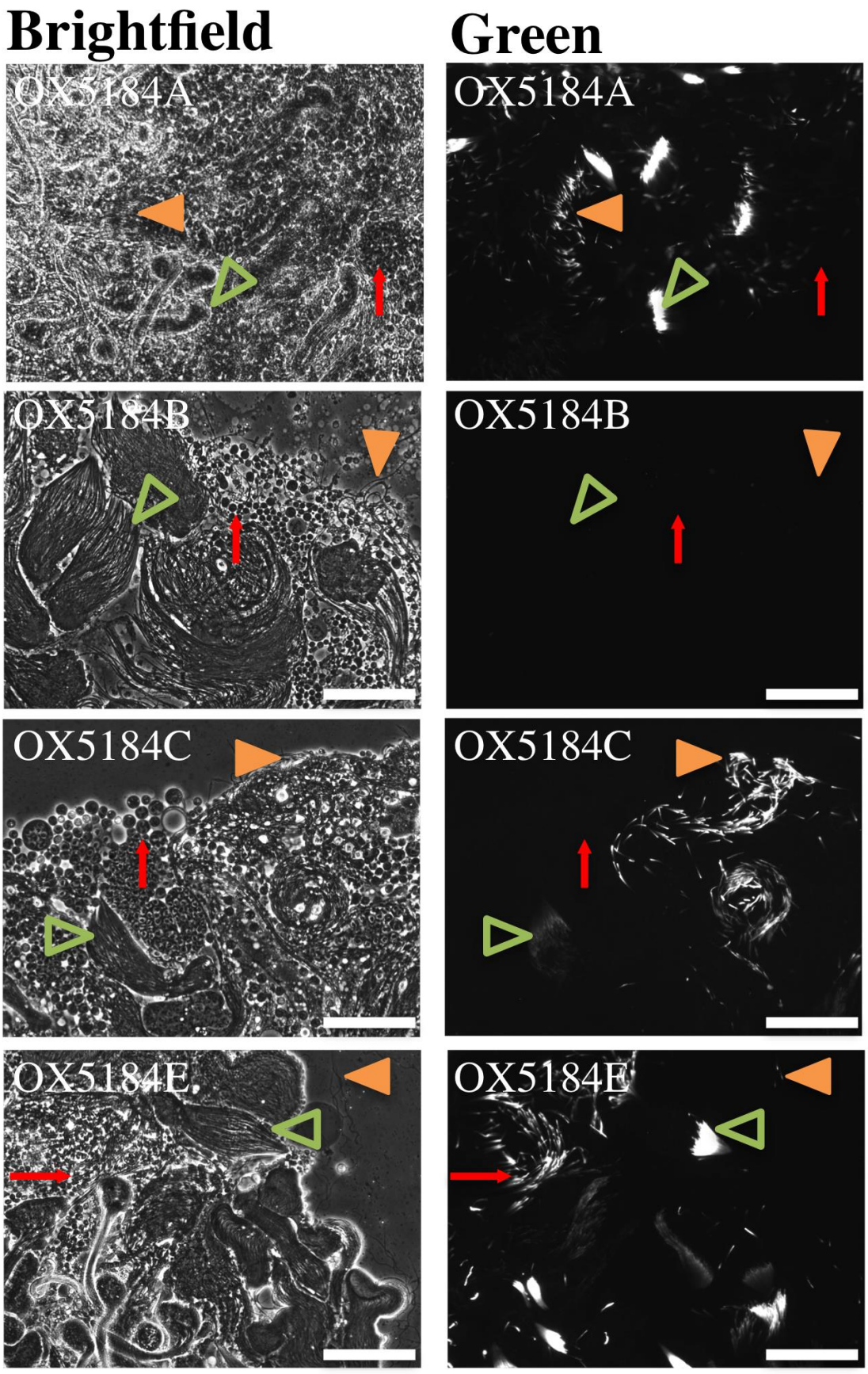


Figure 4.13. DmHsp70 promoter [+21 bp hsp70 5'UTR]-Ccprot1-zsGreen (OX5184) mediates reporter expression in the male germline in of five of six tested lines (OX5184A, C, E, F & K) [continued on next page]. Fluorescence and phase contrast microscopy of Medfly testes demonstrates fluorescent reporter localisation in sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), of most lines with visible reporter expression. Scale bars: 100 µm.

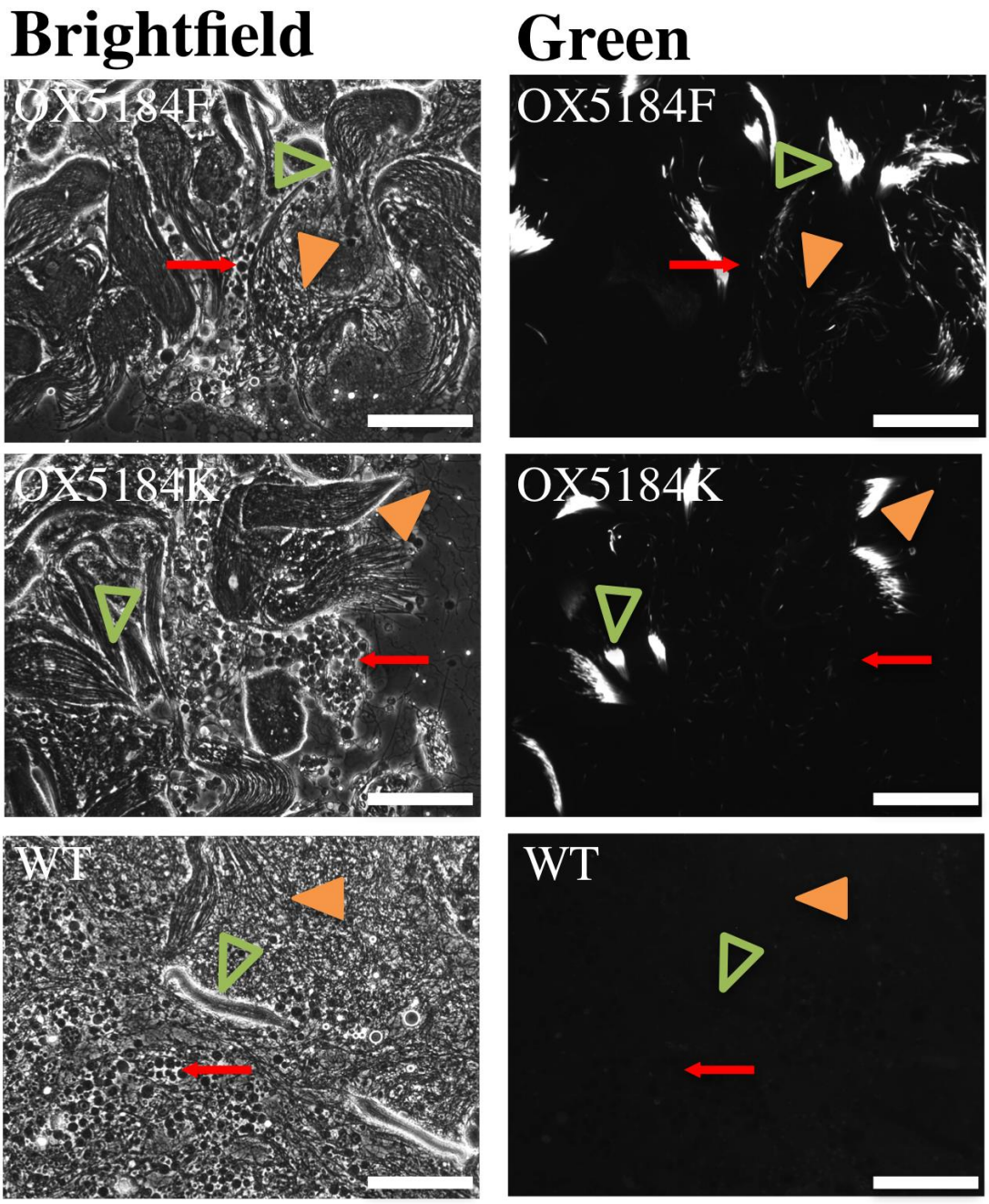


Figure 4.13. DmHsp70 promoter [+21 bp hsp70 5'UTR]-Ccprot1-zsGreen (OX5184) mediates reporter expression in the male germline in of five of six tested lines (OX5184A, C, E, F & K). Fluorescence and phase contrast microscopy of Medfly testes demonstrates fluorescent reporter localisation in sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), of most lines with visible reporter expression. Scale bars: 100 μm.

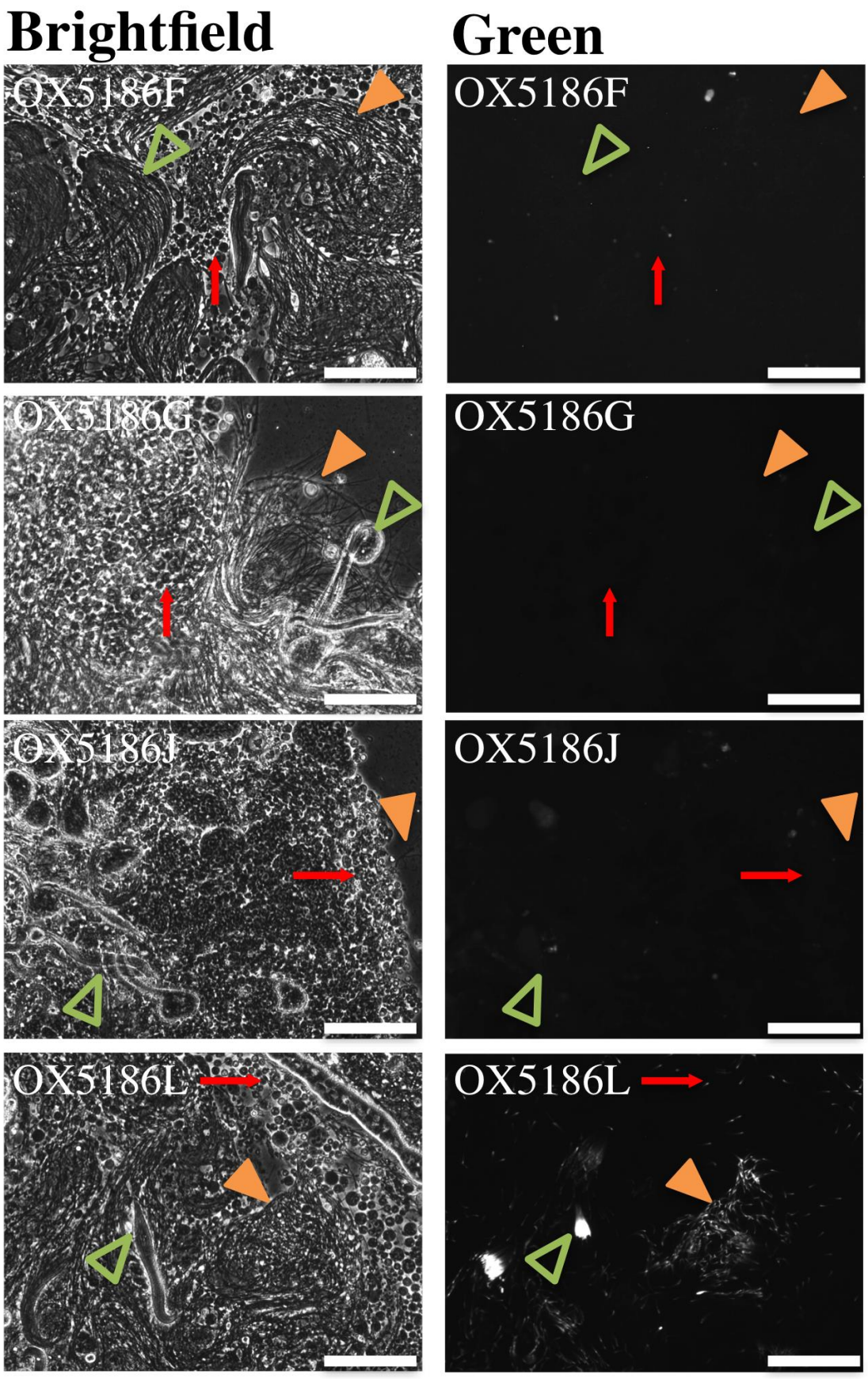


Figure 4.14. DmHsp70 promoter [+31 bp hsp70 5'UTR]-Ccprot1-zsGreen (OX5186) mediates reporter expression in the male germline of three of six tested lines (OX5186L, M & N) [continued on next page]. Fluorescence and phase contrast microscopy of Medfly testes demonstrates fluorescent reporter localisation in sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), of most lines with visible reporter expression. Scale bars: 100 µm.

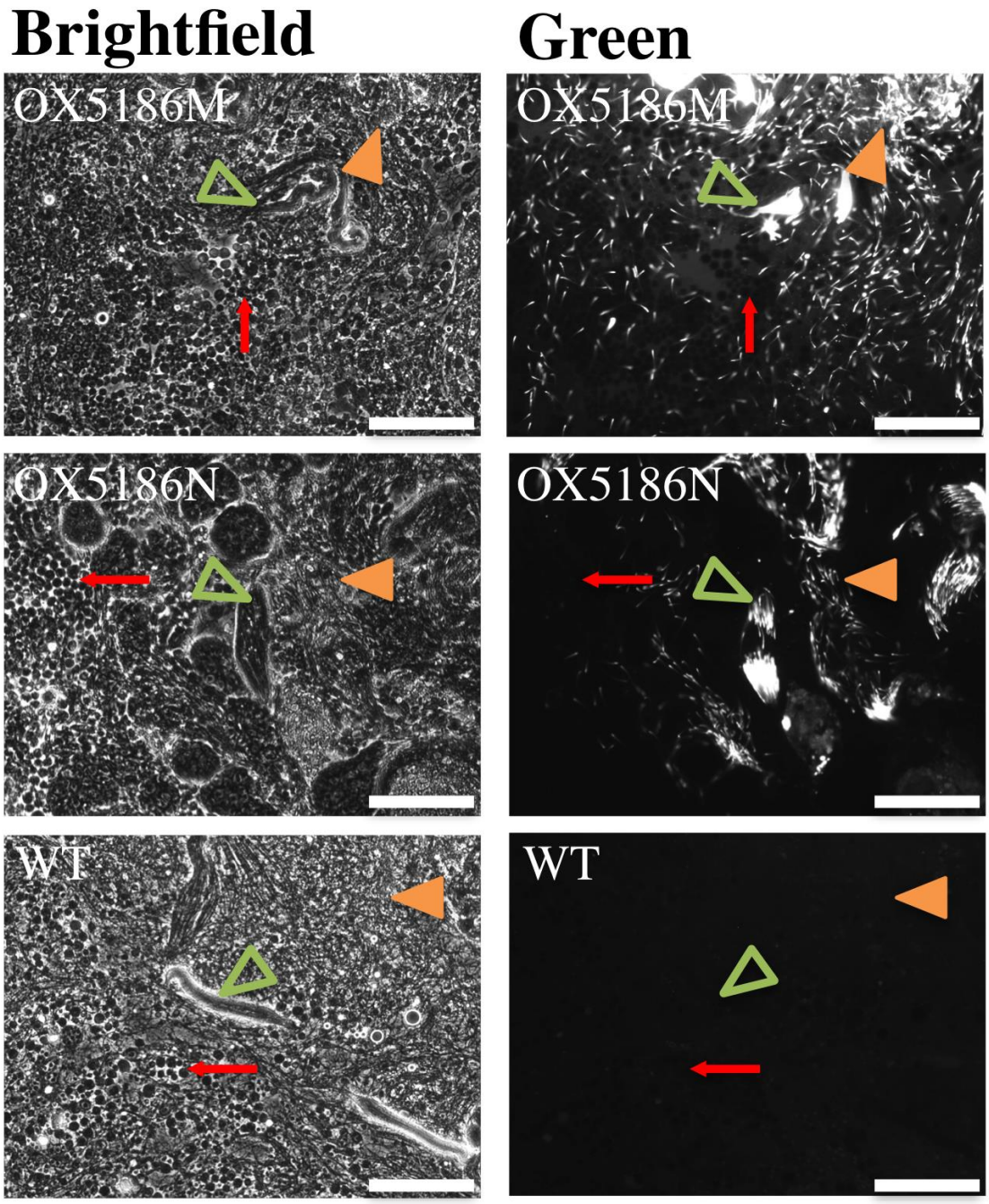


Figure 4.14. DmHsp70 promoter [+31 bp hsp70 5'UTR]-Ccprot1-zsGreen (OX5186) mediates reporter expression in the male germline of three of six tested lines (OX5186L, M & N). Fluorescence and phase contrast microscopy of Medfly testes demonstrates fluorescent reporter localisation in sperm (shaded arrowhead) and spermatids (unshaded arrowhead), but not spermatocytes (arrow), of most lines with visible reporter expression. Scale bars: 100 µm.

4.5 Conclusions

The present study intended to improve upon previous designs for a transgenic system expressing effectors for repressible male sterility and fluorescent sperm marking. The tetO-Dmprot2-FokI system mediated repressible sterility reproducibly (OX4353, OX4718, and OX5036). However, investigation of Dmhsp70-Dmprot2-mCherry-FokI expression and sperm transfer to mated females (OX4718A) indicated that its translation was earlier than desired, and reduced the ability of sperm to behave normally in the female reproductive tract **(Figures 3.5 and 3.8**). One, or both, of the following were considered likely to have mediated this effect. First, the effector featured a partial 5' UTR (89 bp) from Dmhsp70, a gene with embryonic translation, preceding the Dmprot2 5'UTR (Dmhsp70 5'UTR-Dmprot2 5'UTR). This might have contributed to early translation, which the inclusion of the Dmprot2 5' UTR was intended to prevent. Alternatively, it was possible that the Dmprot2 5'UTR did not mediate a translational delay in Medfly equivalent to that observed in *D. melanogaster* (Jayaramaiah Raja and Renkawitz-Pohl, 2005), (Barckmann et al., 2013). Therefore, we reasoned that translation of the male sterility effector could be appropriately delayed by replacing the chimeric Dmhsp70 5'UTR-Dmprot2 5'UTR with a single 5'UTR from a Dmprot gene or, more preferably, its homologues in Medfly. Similarly, a suitable fluorescent sperm marking system could be engineered by placing a fluorescent marker under the transcriptional and translational control of these protamine-like genes, as a module independent of the male sterility effector.

We therefore investigated the production and localisation of a reporter, regulated by three new protamine sequences (Ccprot1, Ccprot2 & Dmprot1), in the male germline. Fluorescence was not visible in testes of Dmprot1-zsGreen expressing males (OX5140) of four lines, despite the presence of transcript in OX5140E (the other three lines were not assessed). Because fusions of a fluorescent protein to the C-terminus of the highly similar Dmprot2 protein were previously shown to perform poorly (OX4718/4751/4801), we concluded that this fusion (Dmprot1-zsGreen) may similarly, have been poorly tolerated. However, it could not be excluded that the Dmprot1 5'UTR does not mediate translational repression in Medfly, equivalently to *D. melanogaster*. It should be noted that translational delay was not observed in a Dmprot2-FokI fragment regulated by Dmhsp70 5'UTR-Dmprot2 5'UTR (**Figure 3.5**); though it could not be excluded that the presence of Dmhsp70 5'UTR was responsible for this effect. It would not be possible to conclusively identify which factor was responsible, without

substituting the Dmprot1-zsGreen fusion or the Dmprot1 5'UTR into a different expression system, wherein all other components were shown to mediate reporter localisation to spermatids and sperm. For instance, assessing the zsGreen localisation mediated by a Ccprot1 promoter-Dmprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1 3'UTR fragment, would confirm if the Dmprot1 5'UTR was capable of mediating translational delay.

Despite the failure of Dmprot1-zsGreen (OX5140) to regulate a transgenic effector in the male germline appropriately, three other systems were successful: Ccprot1-zsGreen (OX5122), Ccprot1-mCherry (OX5123) and Ccprot2-zsGreen (OX5150). All mediated strong fluorescent reporter localisation to sperm and spermatids, but not spermatocytes. In addition to confirming their functionality as fluorescent sperm marking systems, this indicated that a modification of these transgenic systems could mediate the appropriate translational delay of a protamine-FokI effector, until the elongated spermatid stage. To facilitate the design of such an effector, the TSS of Ccprot1 and Ccprot2 were investigated by 5'RACE and analysis of NCBI RNA-seq data; this would allow the effector to be modified for tetracycline-repressible expression, as previously described (**Section 4.4**). A single TSS was not clearly defined for either of Ccprot1 or Ccprot2; we therefore selected the most upstream potential TSS (as indicated by 5'RACE or high-throughput transcript sequencing), to ensure that all elements likely to mediate the observed translational delay were retained.

That this Ccprot1 5'UTR sequence was sufficient to mediate translational delay, was indicated by the observation that a Dmhsp70 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1 3'UTR fragment retained localisation of zsGreen to elongating spermatids and sperm (OX5184). Notably, the Dmhsp70 promoter fragment had been truncated, retaining 21 bp of Dmhsp70 5'UTR (relative to the 89 bp in OX4353/OX4718). Therefore, this alteration removed the hypothetical ability of the Dmhsp70 5'UTR to confer early translation, without abolishing transcription. This provided evidence that the fragment was suitable to regulate an effector of male sterility, which was assessed in the subsequent chapter, in expression constructs including the Ccprot1-zsGreen fluorescent sperm marking system as a separate module.

We assessed the performance of the Ccprot1-zsGreen fluorescent sperm marking

system independently of a male sterility effector, to avoid the potentially confounding effects of protamine-FokI expression on sperm. There were no obvious effects of Ccprot1-zsGreen expression on male mating competitiveness in OX5122M heterozygotes or homozygotes, which were equally competitive with wild-type in lab-scale testing. Furthermore, in double mating experiments, the genotype of the male to which females were first mated (WT or OX5122M) did not appear to have any effect on the desire of females to re-mate. However, the sample size was quite small because most females (46/60), did not choose to re-mate. Although these tests were a promising indication that the transgenic system performed well, the limitations of lab-scale mating competition assays should be considered, to assess their ability to reflect the complex biology of mating in Medfly.

First, laboratory rearing affects the mating behaviour of males, in particular reducing the duration of key courtship activities during lekking; these are associated with reproductive success (Liimatainen et al., 1997), (Briceño and Eberhard, 1998). The high densities of lab conditions have also been suggested to habituate males to close contact, making them less aggressive, and potentially less dominant for mates (Briceño et al., 1999); though the role of aggression in reproductive success has been contested (Whittier et al., 1992). Interestingly, the composition of emitted male pheromones also differs in mass-reared and wild individuals (Vaníčková et al., 2012). As the OX5122M line is derived from the wild-type background and reared under equivalent conditions, males of both genotypes would be expected to exhibit similar changes in terms of behaviour and semiochemical composition. Therefore, the test would not highlight potential differences in factors that influence mating competitiveness, which are related to mass-rearing or laboratory colonisation, but are independent of the transgene. Furthermore, laboratory cages are not an accurate simulation of the field, which present several stimuli that influence mating behaviour. For instance, vegetation typically provides an environment around which males congregate, to form the lek and compete for females (Prokopy and Hendrichs, 1979). We have observed lekking behaviour in laboratory cages, but in many instances, males who scramble for females without lekking appear to secure mating events. This behaviour, which is substantially different to the circumstances observed in the field, has been reported elsewhere (Mossinson and Yuval, 2003). Additionally, aromatic compounds are present in fruit around which Medfly congregate; these are attractive, and some been shown to affect the reproductive success of males (Shelly et al., 2004), (Papadopoulos et al., 2006). Therefore, after a

candidate strain with a suitable male sterility and fluorescent sperm marking phenotype is isolated, it should be tested under field-like conditions, in greenhouses with natural light, vegetation, low population densities, and with competition against wild-caught males. However, an assessment of this rigour would be unnecessary at this stage, and waste resources that could be applied to develop the necessary transgenic strain incorporating repressible male sterility and fluorescent sperm marking.

We gathered further evidence to validate the ability of the Ccprot1-zsGreen fluorescent sperm marking system, to assess the mating ability of transgenic males in the field. This would be highly useful to assess whether males were competitive for mating events in the field. Females mated twice (to WT and OX5122 males) contained visible sperm of both genotypes (fluorescently marked and non-marked), indicating that the system could be used to score multiple mating events in the field. However, a more detailed analysis should be performed when the system is combined with an effector for repressible male sterility, ideally with a more quantitative and less biased mode of scoring (qPCR or fluorescence quantification, with double blind scoring). High rates of sperm transfer were observed in four transgenic lines (OX5122D, G, K & M), indicating that the system would be likely to function reproducibly for most autosomal insertions. It was also promising that the quantity and quality of sperm transferred to females upon mating, did not appear to vary between OX5122M heterozygotes and homozygotes, relative to WT. Furthermore, the fluorescence of sperm transferred to WT females by OX5122M homozygous males persisted for at least two weeks after death on a sticky trap, and facilitated differential scoring of the male genotype to which females had mated (wild-type or OX5122M), with > 90% accuracy. As a whole, these results indicated that the mating ability of OX5122 males was relatively similar to wild-type, despite expression of the transgenic marker system. However, it will be interesting to determine if these conclusions remain valid, when a repressible male sterility effector is expressed within the same expression construct; this was investigated in the subsequent chapter.

Chapter 5 – Characterisation of transgenic lines with penetrant and repressible male sterility and fluorescent sperm marking

5.1 Single expression constructs for repressible male sterility and fluorescent sperm marking (OX5195, OX5241, OX5242 & OX5257)

The prior study (Chapter 4) demonstrated that a suitable fluorescent marking system could be engineered by placing a fluorescent reporter (zsGreen or mCherry) under the transcriptional and translational control of Medfly protamine-like genes (protamine promoter-protamine 5'UTR-protamine ORF-fluorescent reporter-protamine 3'UTR). The Ccprot1-ZsGreen system (OX5122) performed best; strong zsGreen localisation was observed in the nuclei of sperm and spermatids (5/5 lines). The Ccprot1-mCherry (OX5123) and Ccprot2–zsGreen systems (OX5150) performed acceptably, but appeared more sensitive to positional effect (not all insertions demonstrated fluorescent reporter localisation in nuclei of spermatids and sperm). Therefore, it appeared that the Ccprot1-zsGreen (OX5122), Ccprot1-mCherry (OX5123), or Ccprot2-zsGreen (OX5150) systems could be modified to appropriately regulate the effector of repressible male sterility, engineering a translational delay to the elongated spermatid stage.

This was important, because translational repression was not observed in the prior male sterility system (OX4718: **Figure 3.5** and **Figure 3.8**). We subsequently demonstrated that a Dmhsp70 promoter-Ccprot1 5'UTR-zsGreen-Ccprot1 3'UTR fragment (truncated to remove all but 21 bp of the predicted Dmhsp70 5'UTR), was able to specifically localise the zsGreen reporter to elongating spermatids and mature sperm. Therefore, it appeared that this system could potentially enhance the mating competitiveness of an engineered line, facilitating a reduced scale of sterile male release.

However, it was still necessary to adapt the Ccprot1 and Ccprot2 sequences to regulate the effector of male sterility in a tetracycline-repressible manner (**Figure 5.1**). First, substitution of FokI for zsGreen/mCherry was required, to replace sperm marking with protamine-mediated cleavage of the sperm genome. Thereafter, integration with the previously described, tetracycline-repressible male germline switch (tetO21-Ccβ2T-Cchsp83-tTAV) to provide repressible sterility was necessary. This involved placement of a Ccprot1-FokI or Ccprot2-FokI system in a head-to-head configuration to the male

germline switch, to put the tetO21 site upstream of the Ccprot-Fok1 transcription unit, and thus facilitate tetracycline repression. Finally, substitution of the Ccprot1 or Ccprot2 promoter for a minimal promoter fragment (from Dmhsp70) was required, for compatibility with the tet-off system. To facilitate ligation of tetO-Dmhsp70 minimal promoter to Ccprot1 or Ccprot2 5'UTR without disrupting the regulatory capacity of each component, we selected the most conservative (longest) 5'UTR, as indicated by 5' RACE, or the high throughput transcript sequencing data available from NCBI. It remained possible that the expression profile of the effector would be modified, when it was combined with the tetracycline-repressible system. Four candidates were generated, which featured the Ccprot1-zsGreen system for fluorescent sperm marking, and one of four effectors of repressible male sterility.

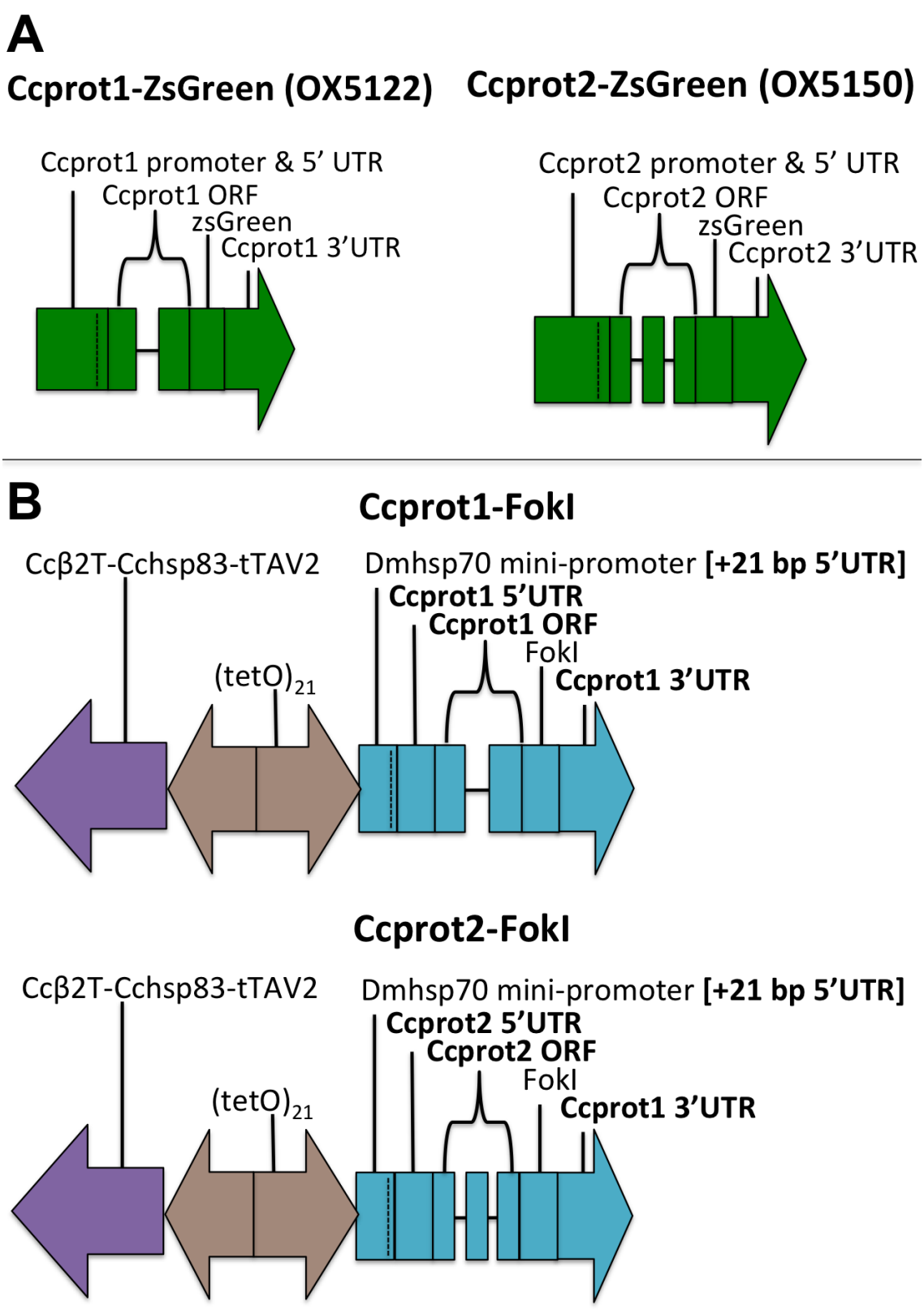
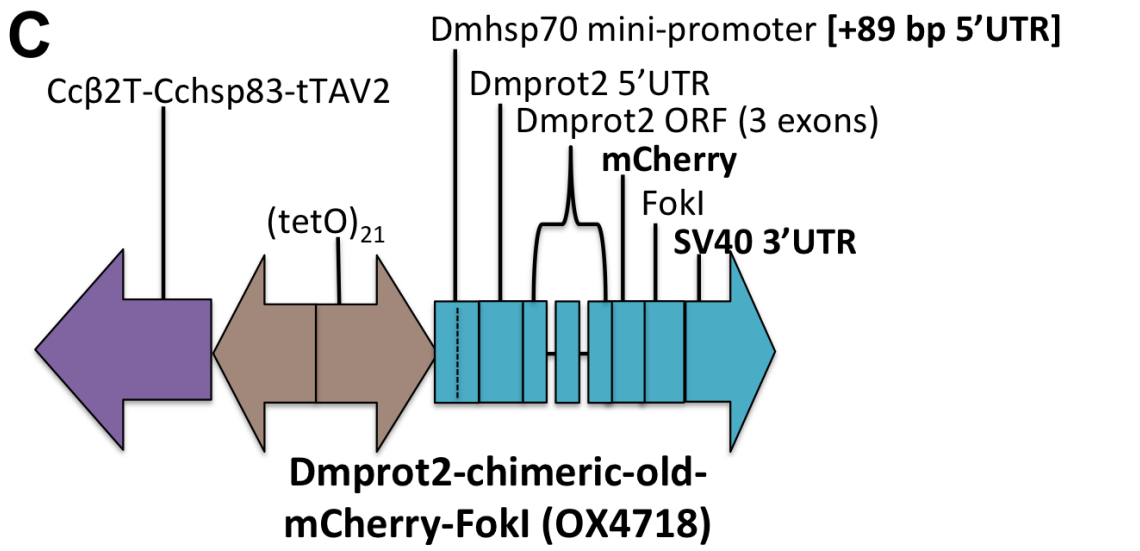
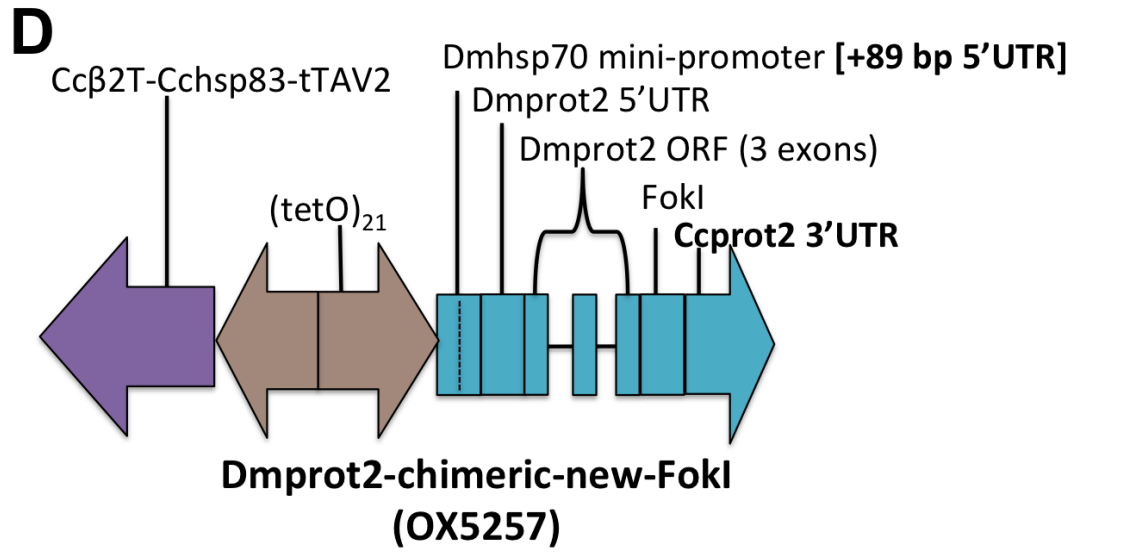


Figure 5.1. Adaptation of Medfly protamine-like genes to regulate effectors of repressible male sterility (continued on next page). (**A**) Ccprot1-zsGreen & Ccprot2-zsGreen were confirmed to localise ZsGreen to elongating spermatids and sperm (Chapter 4). The unmodified Ccprot1-zsGreen fragment was applied for fluorescent sperm marking. Dashes indicate promoter-5'UTR boundary. (**B**) Modification of Ccprot1-ZsGreen and Ccprot2-zsGreen to regulate effectors of repressible male sterility: protamine promoter was replaced with Dmhsp70 minimal promoter; Dmhsp70 5'UTR elements from the prior system (OX4718) were trimmed (89 bp → 21 bp); and this fragment was attached to the previously described tetracycline-repressible transcriptional switch (Ccβ2T-Cchsp83-tTAV).





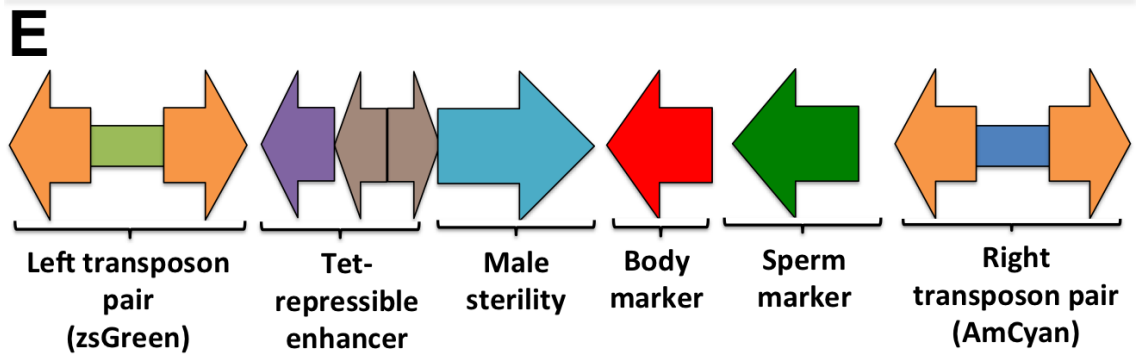


Figure 5.1. Adaptation of Medfly protamine-like genes to regulate effectors of repressible male sterility. (**C-D**) Male sterility effector from OX4718 (**C**), modified for use in OX5257 (**D**). This construct incorporates a longer Dmhsp70 5'UTR fragment (89 bp), relative to Ccprot1-FokI or Ccprot2-FokI. (**E**) Simplified 4-ended piggyBac vector used for all constructs in this chapter. Full expression construct diagrams are provided in the next figure.

The first candidate (OX5195) applied Ccprot1 and Dmhsp70 elements for repressible male sterility, largely as previously described (tetO21-Dmhsp70 minipromoter-Ccprot1 5'UTR-Ccprot1 ORF-FokI-Ccprot1 3'UTR; hereafter Ccprot1-full 5'UTR-FokI). The construct mediated highly penetrant male sterility, but was not repressible (**Section 5.2**). We concluded that irrepressibility potentially resulted from a minimal promoter within the predicted Ccprot1 5'UTR, circumventing tetracycline-mediated repression. Consequently, we shortened the predicted 5'UTRs of Ccprot1 and Ccprot2 to attempt removal of non-repressible elements, and investigated their functionality in equivalent expression systems: OX5241 (Ccprot2-short 5'UTR-FokI) and OX5242 (Ccprot1-short 5'UTR-FokI). These were evaluated in Sections 5.3 and 5.4, respectively.

A backup was required to mediate the risk of sequence elements behaving in unpredicted ways. The Dmprot2-chimeric-old-mCherry-FokI effector (OX4718, **Figure 3.5**) mediated penetrant and repressible male sterility. We reasoned that fluorescent sperm marking could be engineered by addition of Ccprot1-ZsGreen (validated in OX5122). However, a high rate of transfer of morphologically normal sperm appeared unlikely, if the expression system behaved consistently in all penetrant lines (it was previously demonstrated that sperm of one line were negatively affected by Dmprot2-chimeric-old-mCherry-FokI expression; **Figure 3.8**). The effector was essentially unmodified, to reduce the risk of altering the previously characterised phenotype, though two changes were performed: (1) mCherry was removed (inclusion of a semi-functional marker would complicate licensing) and (2) the SV40 3'UTR was substituted for Ccprot2 3'UTR (a marketing decision, to avoid inclusion of virally derived elements). It should be noted that a single copy of SV40 3'UTR was retained as a regulator of zsGreen, used to visually monitor excision of one *piggyBac* end pair. This was not considered to be problematic, because the sequence would be absent in the final product (following *piggyBac* end removal). The longer, partial Dmhsp70 5'UTR (89 bp; 21 bp in all other constructs) of the parent molecule was retained. Alteration of these elements (mCherry and SV40 3'UTR) could theoretically modify the phenotype, either by alteration of translational stability (the novel Ccprot2 3'UTR) or the structure of the effector molecule (mCherry removal). Neither was considered likely, because constructs applying Dmprot2-chimeric-FokI without mCherry were penetrant and repressible previously (eg. OX4353/5036); and because a generic 3'UTR is usually sufficient to facilitate appropriate translation in the male germline of *D. melanogaster* (Michiels et

al., 1989), (Jayaramaiah Raja and Renkawitz-Pohl, 2005). The resulting effector of male sterility was termed Dmprot2-chimeric-new-FokI (OX5257).

All constructs (**Figure 5.2**) were 4-ended *piggyBac* vectors incorporating resolvable ends (two pairs of *piggyBac* ends with fluorescent markers); fluorescent sperm marking (Ccprot1-zsGreen); a transformation marker brightly expressed in muscle tissue (mActin-dsRed2); and a tetracycline repressible transcriptional activator in the male germline (Ccβ2T-Cchsp83-tTAV2) that regulates the tetO21-protamine-FokI male sterility effector. Difficulty sequencing adjacent repetitions of Cchsp83 3'UTR (OX5195), which were associated with (1) the zsGreen fluorescent marker in the left-handed *piggyBac* pair and (2) tTAV2, indicated secondary structure that could prevent excision of *piggyBac* ends. For this reason, one Cchsp83 3'UTR copy was substituted in subsequent constructs (ZsGreen-Cchsp83 3'UTR → ZsGreen-SV40 3'UTR).

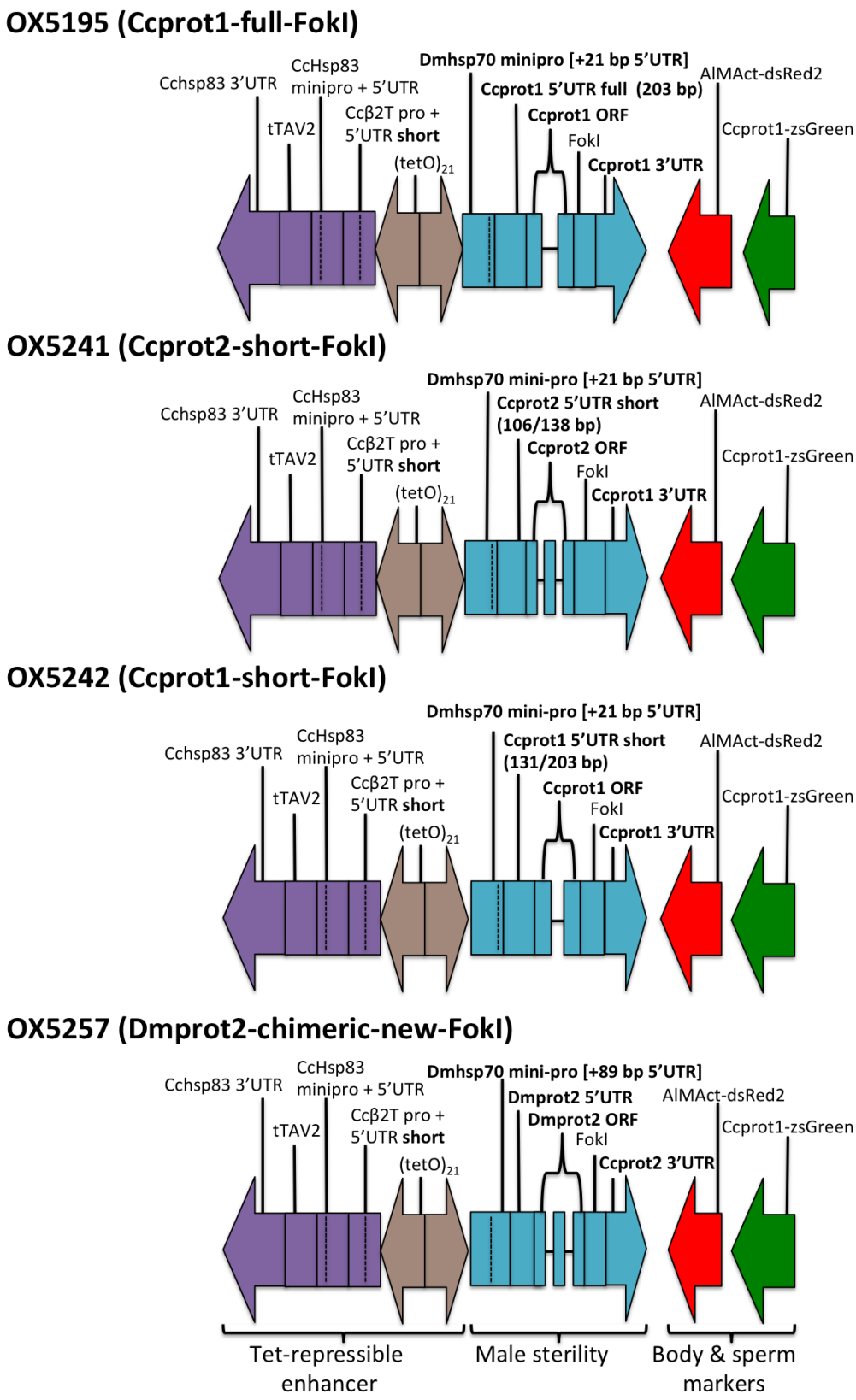


Figure 5.2. Expression constructs for tetracycline-repressible male sterility and fluorescent sperm marking. Four effectors of repressible male sterility were tested: OX5195 (Ccprot1-full 5' UTR-FokI); OX5241 (Ccprot2-short 5'UTR-FokI); OX5242 (Ccprot1-short 5'UTR-FokI); OX5257 (Dmprot2-chimeric-new-FokI). Full and short indicate 5'UTR length. Dashes indicate promoter-5'UTR boundary. The failsafe construct (OX5257) incorporated a repressible male sterility effector similar to a prior design, OX4718. The fluorescent body marker (AlMAct promoter-AlMAct 5' UTR-dsRed2-AlMAct 3' UTR) and sperm marker (Ccprot1 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1 3'UTR) were as previously described. Note: piggyBac ends omitted from these four-ended vectors (full diagrams shown in Figure 2.2).

5.2 OX5195 (Ccprot1-full 5'UTR-FokI) males are irrepressibly sterile in penetrant insertions

5.2.1 Establishment of OX5195 transgenic lines

Microinjection (**Table 5.1**), backcrossing to WT (**Table 5.2**), screening for transient expression of the transformation marker, and Mendelian assessment of insertions were generally as previously described. Buffered injection mixes were prepared with tetracycline (100 ng/µl), OX5195 (600 ng/µl), mRNA helper OX3081 and DNA helper OX3022 (300 ng/µl each). Transient AlMAct-dsRed2 expression was observed in G_0 pupae, indicating successful microinjection. Adult survival (32%) was similar to typical results for injection of Medfly (25%) (Gregory et al., 2016). Two rounds of injection were performed, denoted (1) or (2). Transformation efficiency was poor (1.2%), despite large adult G0 cohorts (n=1182).

Table 5.1: Microinjection logistics for OX5195

Round	Embryos	Larvae	Pupae	Adults	Lines
1	1343	981 (73%)	143 (11%)	122 (9%)	2 (1.6%)
2	2360	1410 (60%)	1114 (47%)	1060 (45%)	13 (1.2%)
Total	**3703**	**2391 (65%)**	**1257 (34%)**	**1182 (32%)**	**15 (1.3%)**

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (transgenic lines/G_0 adults crossed).

Table 5.2: G_0 backcrosses to establish OX5195 lines

OX5195(1)A	10 ♀ x 10 WT ♂	OX5195(1)B	12 ♀ x 10 WT ♂	**OX5195(1)C**	**12 ♀ x 10 WT ♂**
OX5195(1)D	13 ♀ x 10 WT ♂	**OX5195(1)E**	**11 ♀ x 10 WT ♂**	OX5195(1)F	4 ♀ x 4 WT ♂
OX5195(2)A	10 ♂ x 30 WT ♀	OX5195(2)B	10 ♂ x 30 WT ♀	**OX5195(2)C**	**23 ♀ x 10 WT ♂**
OX5195(2)D	10 ♂ x 30 WT ♀	OX5195(2)E	11 ♂ x 30 WT ♀	OX5195(2)F	11 ♂ x 30 WT ♀
OX5195(2)G	11 ♂ x 30 WT ♀	**OX5195(2)H**	**23 ♀ x 10 WT ♂**	OX5195(2)I	10 ♂ x 30 WT ♀
OX5195(2)J	10 ♂ x 30 WT ♀	OX5195(2)K	10 ♂ x 30 WT ♀	OX5195(2)L	10 ♂ x 30 WT ♀
OX5195(2)M	10 ♂ x 30 WT ♀	OX5195(2)N	10 ♂ x 30 WT ♀	OX5195(2)O	10 ♂ x 30 WT ♀
OX5195(2)P	**22 ♀ x 10 WT ♂**	OX5195(2)Q	22 ♀ x 10 WT ♂	OX5195(2)R	11 ♂ x 30 WT ♀
OX5195(2)S	11 ♂ x 30 WT ♀	OX5195(2)T	11 ♂ x 30 WT ♀	OX5195(2)U	11 ♂ x 30 WT ♀
OX5195(2)V	11 ♂ x 30 WT ♀	OX5195(2)W	11 ♂ x 30 WT ♀	OX5195(2)X	11 ♂ x 30 WT ♀
OX5195(2)Y	10 ♂ x 30 WT ♀	OX5195(2)Z	21 ♀ x 10 WT ♂	OX5195(2)AA	21 ♀ x 10 WT ♂
OX5195(2)AB	21 ♀ x 10 WT ♂	**OX5195(2)AC**	**21 ♀ x 10 WT ♂**	OX5195(2)AD	10 ♂ x 30 WT ♀
OX5195(2)AE	10 ♂ x 30 WT ♀	OX5195(2)AF	10 ♂ x 30 WT ♀	OX5195(2)AG	10 ♂ x 30 WT ♀
OX5195(2)AH	10 ♂ x 30 WT ♀	**OX5195(2)AI**	**10 ♂ x 30 WT ♀**	OX5195(2)AJ	10 ♂ x 30 WT ♀
OX5195(2)AK	10 ♂ x 30 WT ♀	OX5195(2)AL	10 ♂ x 30 WT ♀	OX5195(2)AM	10 ♂ x 30 WT ♀
OX5195(2)AN	10 ♂ x 30 WT ♀	OX5195(2)AO	21 ♀ x 10 WT ♂	OX5195(2)AP	21 ♀ x 10 WT ♂
OX5195(2)AQ	21 ♀ x 10 WT ♂	**OX5195(2)AR**	**21 ♀ x 10 WT ♂**	OX5195(2)AS	21 ♀ x 10 WT ♂

OX5195(2)AT	21 ♀ x 10 WT ♂	**OX5195(2)AU**	**20 ♀ x 10 WT ♂**	OX5195(2)AV	20 ♀ x 10 WT ♂
OX5195(2)AW	**20 ♀ x 10 WT ♂**	OX5195(2)AX	20 ♀ x 10 WT ♂	**OX5195(2)AY**	**11 ♂ x 30 WT ♀**
OX5195(2)AZ	11 ♂ x 30 WT ♀	**OX5195(2)BA**	**11 ♂ x 30 WT ♀**	**OX5195(2)BB**	**11 ♂ x 30 WT ♀**
OX5195(2)BC	11 ♂ x 30 WT ♀	OX5195(2)BD	11 ♂ x 30 WT ♀	OX5195(2)BE	11 ♂ x 30 WT ♀
OX5195(2)BF	11 ♂ x 30 WT ♀	**OX5195(2)BG**	**11 ♂ x 30 WT ♀**	OX5195(2)BH	10 ♂ x 30 WT ♀
OX5195(2)BI	11 ♂ x 30 WT ♀	OX5195(2)BJ	11 ♂ x 30 WT ♀	**OX5195(2)BK**	**11 ♂ x 30 WT ♀**
OX5195(2)BL	11 ♂ x 30 WT ♀	OX5195(2)BM	11 ♂ x 30 WT ♀	OX5195(2)BN	11 ♂ x 30 WT ♀
OX5195(2)BO	10 ♂ x 30 WT ♀	OX5195(2)BP	18 ♀ x 10 WT ♂	OX5195(2)BQ	17 ♀ x 10 WT ♂
OX5195(2)BR	17 ♀ x 10 WT ♂	OX5195(2)BS	88 ♂ x 25 ♀		

Underlined crosses gave transgenic lines. The OX5195(2)BS cross used male and female survivors.

5.2.2 OX5195 transgenic G_1 male individuals are frequently infertile

Only data from OX5195(2) lines are presented in detail, because OX5195(1) injections generated only two lines. One was impenetrant (OX5195(1)C); the other (OX5195(1)E) was lost because male transgenics were infertile, even when reared on-tet. Thirteen OX5195(2) lines were derived, facilitating a thorough phenotypic analysis that accounted for potential male irrepressibility. Where possible, tet-reared G_1 transgenic males (1 x 5 WT) and females (1 x 2 WT) of each pool were independently backcrossed to prevent line loss (in case males were infertile, even in the presence of tetracycline in the larval diet). Fertility was preliminarily assessed by larval growth on diet for males; for females, surplus individuals were filtered (day 10) and assessed under egg hatch assay conditions. WT females mated to transgenic males were qualitatively scored for marked sperm (day 8) under the Oxitec microscope setup (Motic BA210 microscope, Fraen fluorescence FLUOLED lamp, Lumenera Infinity 2 camera, at 10x magnification).

Transgenic G_1 male individuals were frequently infertile, indicating male-specific non-repressibility of the sterility phenotype (**Table 5.3**). Individuals from 11/13 pools (n=34) were assessed. G_1 transgenic males were observed in 6/11 pools. Males of four pools were sterile (OX5195(2)P, AC, AU & AW); males of two pools were fertile (OX5195(2)AI & BA). Female individuals were fertile in all pools (9/9). Male infertility and sperm marking were correlated; infertile males produced sperm with visible fluorescence. This suggested that fluorescent sperm marking in G_1 individuals can be used as a preliminary screen for penetrant lines (**Figure 5.3**); this was recapitulated in subsequent analyses (**Sections 5.3-5.5**).

Table 5.3 Infertility of male OX5195(2) G_1 individuals indicates that protamine-FokI expression is frequently non-repressible

G1 pool	Fertility				Sperm marking	Apparent phenotype
	Male		Female			
	%	n	%	n		
OX5195(2)P	0	1	100	1	Yes	Penetrant but non-repressible male sterility with fluorescent sperm marking
OX5195(2)AC	0	1	100	3	Yes	
OX5195(2)AU	0	4	100	6	Yes	
OX5195(2)AW	0	2			Yes	
OX5195(2)AI	100	2	100	2	No	Impenetrant, no fluorescent sperm marking
OX5195(2)BA	100	2			No	
OX5195(2)H			100	3		Male G_1 individuals were not present
OX5195(2)AR			100	2		
OX5195(2)AY			100	6		
OX5195(2)BK			90	10		
OX5195(2)BG			100	2		

Lines providing only females (OX5195(2)H, AR, AY, BK & BG) could not be assessed for male-specific, non-repressible infertility or sperm marking at this stage. The presence of fluorescent marking in the male germline is assessed in **Figure 5.3**. "n": number of individuals assessed.

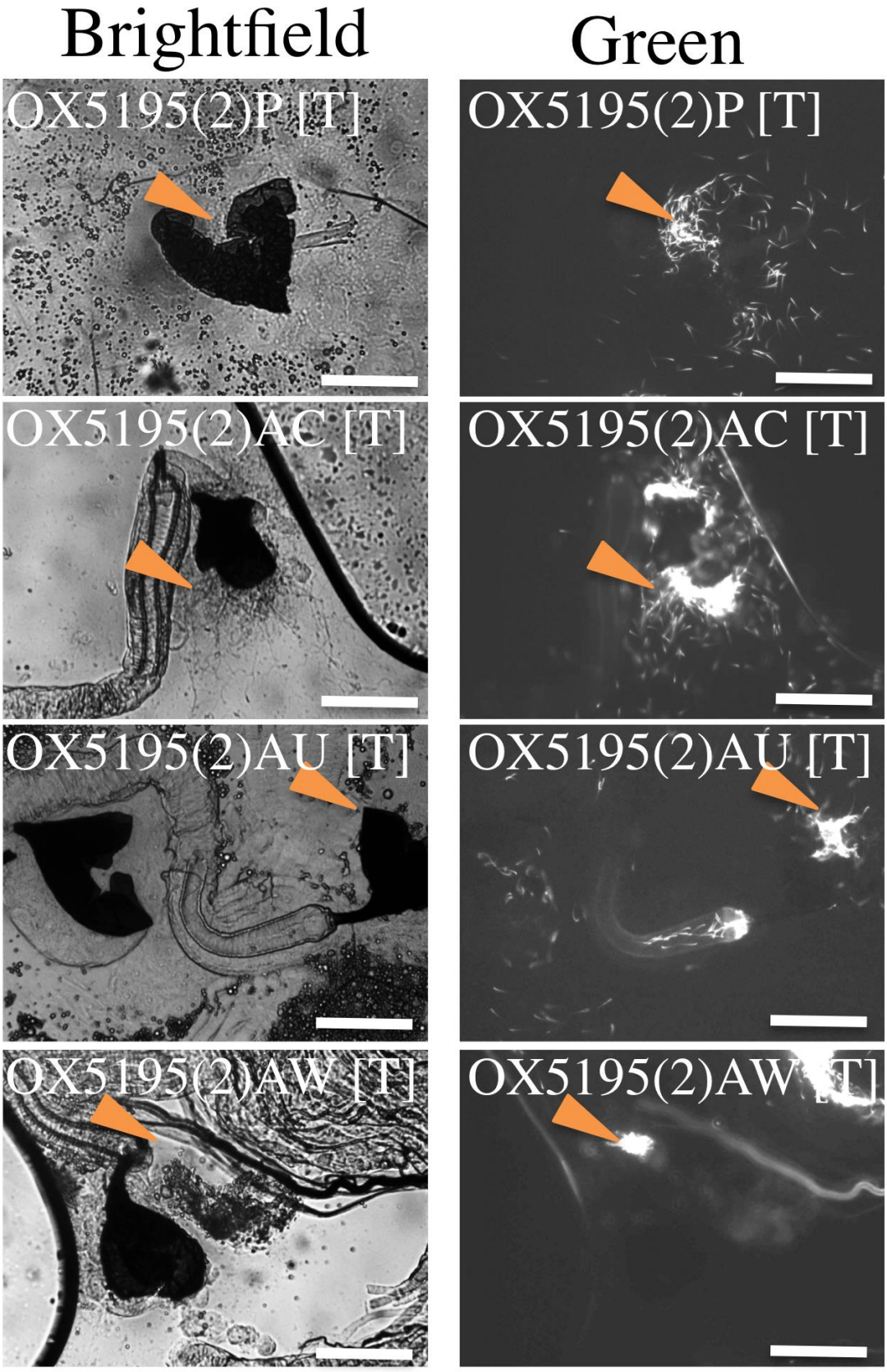


Figure 5.3. Fluorescent marker expression in sperm (Ccprot1-zsGreen) from G_1 transgenic individuals of OX5195 (Ccprot1-full 5'UTR-FokI) is correlated with irrepressible sterility [continued on next page]. OX5195(2) G_1 trangenic males (tet-reared) were mated to WT females; the spermatchecae were dissected. Males of OX5195(2)P, AC, AU & AW transferred marked sperm (arrowheads) to females but were completely infertile. Males of OX5195(2) AI & BA transferred non-marked sperm (arrowheads) and were fertile. Scale bars: 100 µm.

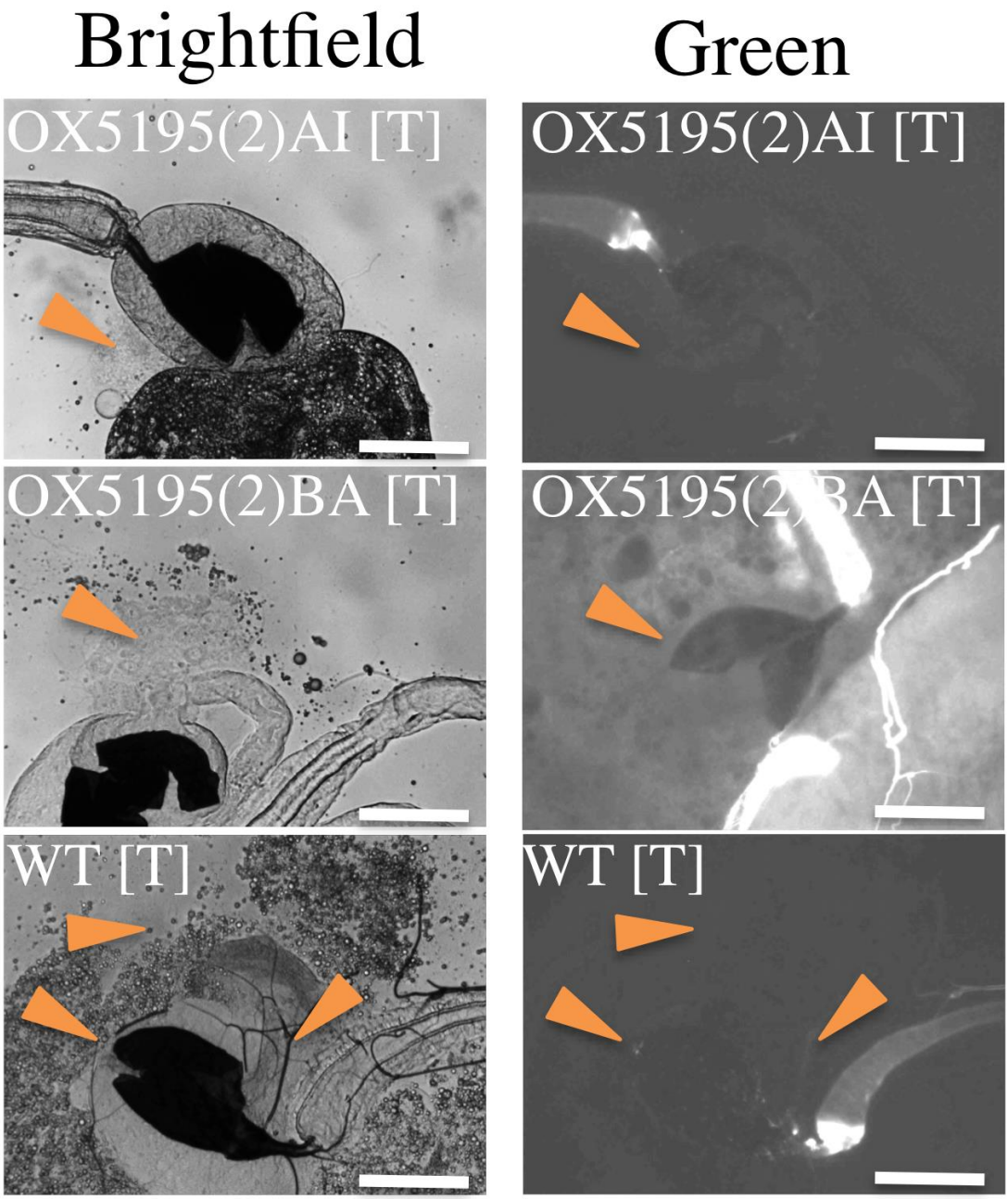


Figure 5.3. Fluorescent marker expression in sperm (Ccprot1-zsGreen) from G_1 transgenic individuals of OX5195 (Ccprot1-full 5'UTR-FokI) is correlated with irrepressible sterility. OX5195(2) G_1 trangenic males (tet-reared) were mated to WT females; the spermatchecae were dissected. Males of OX5195(2)P, AC, AU & AW transferred marked sperm (arrowheads) to females but were completely infertile. Males of OX5195(2) AI & BA transferred non-marked sperm (arrowheads) and were fertile. Scale bars: 100 μm.

Two pools were lost: OX5195(2)AW (no females present; all males were sterile) and OX5195(2)P (the male was sterile and the female cross was not kept), leaving 9 pools. Although the OX5195 system (Ccprot1-full 5'UTR-FokI) was frequently non-repressible, we continued analysis because it was theoretically possible to recover repressible insertions from pools where males were fertile (OX5195(2)AI & BA) or not yet analysed (OX5195(2)H, AR, AY, BK, BG). Furthermore, it was preferable to confirm that non-repressibility was reproducible by replicated egg hatch assay; and to investigate the effect of Ccprot1-FokI expression on sperm fitness on a higher resolution microscope (Cardiff University). The rationale was that even if this version of the system was non-functional due to irrepressibility, a novel variant of the Ccprot1-full 5'UTR-FokI system would be engineered, for full repressibility. A single line was established from each pool (generally from a female as males were frequently infertile) and evaluated further. For transgenic G_1 pools where males were infertile, we predicted that males derived from a female transgenic lineage would have a penetrant and non-repressible phenotype. This was because all individuals of the same pool were assumed to possess the same insertion; the transformation efficiency of OX5195 was low.

All lines were single insertions (**Table 5.4**). OX5195(2)BA1 was excluded from further analysis because it was Y-linked (cannot be made homozygous). Sex-linkage was not detected in other lines because transgenic females were used, to circumvent non-repressibility. It was possible to investigate male crosses of lines subsequently demonstrated repressible; it was not pursued because they were not useful commercially (impenetrant).

Table 5.4: Mendelian assessment of OX5195(2) transgenic lines

Line	Sex	Copy number		Sex-linkage		Summary	
		Transgenic (%)	n	Sex ratio (M/F)	n	Copies	Location
OX5195(2)BG1	♀	45	183	0.9	57	1	Unknown
OX5195(2)H1	♀	51	190	1.3	72	1	Unknown
OX5195(2)AI1	♀	54	151	1.0	68	1	Unknown
OX5195(2)BK1	♀	47	136	1.0	58	1	Unknown
OX5195(2)AR1	♀	55	237	1.0	110	1	Unknown
OX5195(2)AU1	♀	53	311	1.1	144	1	Unknown
OX5195(2)AC2	♀	53	207	1.4	90	1	Unknown
OX5195(2)AY1	♀	44	189	2.8	69	1	Unknown
OX5195(2)BA1	♂			Male-only	48	1	Y

The transgenic copy number of OX5195(2)BA1 was not assessed (it was Y-linked and therefore not commercially suitable). "n": number of individuals assessed.

5.2.3 OX5195 lines with penetrant male sterility are not repressible and demonstrate undesirable effects on sperm

Penetrance and repressibility were assessed by egg hatch assay, generally as previously described. F_0 crosses were 10 non-tet [NT] reared WT males to 20 tet-reared [T] heterozygous OX5195(2) females, with F_1 progeny filtered to tet- and non-tet diet. Five F_1 heterozygous males of each group (NT or T) were crossed to ten WT females [NT]. An equivalent cross with WT males [NT] was performed as control. A tet-reared WT control was not performed because prior tests (**Figure 3.6**) demonstrated no significant tetracycline-dependent effect on male viability. However, this was considered to be an oversight, because tetracycline-mediated transgenic repressibility is most accurately represented relative to an equivalent control. Therefore, subsequent assays (OX5241/5242/5257) included a tet-reared WT control (**Sections 5.3-5.5**).

24 hour egg collections (n=100 eggs) were pseudo-duplicated (two replicated measures from the same cage on day 7). Replicates were tested by correction-free chi-square testing for significant variance prior to pooling. Significance was assessed by chi-square testing the number of hatched and unhatched individuals, relative to the WT[NT] control. Penetrance and repressibility were calculated relative to the WT[NT] control. The extent of repressibility was investigated by comparing the number of hatched and unhatched progeny between [NT] and [T] reared males, of each line. Standard error and 95% CI (Wilson) were calculated as recommended for categorical data (Xu et al., 2010). All lines were assessed from single rears over a three day period to minimise environmental or rearing effects.

No lines were commercially applicable (**Table 5.5**). Tetracycline only significantly rescued hatch rate in weakly penetrant lines (**Table 5.6, Figure 5.4**). In the subsequent generation, we investigated the effect of Ccprot1-full 5'UTR-FokI on the quantity of sperm transferred upon mating, under a higher resolution microscope (Cardiff University, Olympus BX50 microscope at 10x or 20x magnification). Only the crosses of non-tet reared transgenic males to WT females were investigated, because a minimal effect of tetracycline for most lines was established, and because we were primarily interested in the effect of Ccprot1-full 5'UTR-FokI at its maximally expressed state (non-repressed). Crosses were performed as described for the egg hatch assay and spermathecae dissected (day 7). Ccprot1-full 5'UTR-FokI expression was associated

with a reduced quantity of sperm transferred to WT females (**Figure 5.5**). Exact quantification was not possible; development of a qPCR assay for quantitative analysis could potentially be beneficial.

Table 5.5 Penetrance and repressibility of male sterility in OX5195(2) lines

Line	**Mean hatch rate (± SE)**		**Penetrance**			**Repressibility**		
	NT	T	%	X^2	$P_{[df]}$	%	X^2	$P_{[df]}$
OX5195(2)AU1	1 ± 0.70	1 ± 0.70	99	177	**< 0.001**$_{[1]}$	1	177	**< 0.001**$_{[1]}$
OX5195(2)AC2	2.5 ± 1.10	3.5 ± 1.30	97	171.2	**< 0.001**$_{[1]}$	4	167.5	**< 0.001**$_{[1]}$
OX5195(2)H1	7 ± 1.80	5 ± 1.54	93	154.9	**< 0.001**$_{[1]}$	5	162.0	**< 0.001**$_{[1]}$
OX5195(2)AI1	63 ± 3.41	89.5 ± 2.17	34	30.9	**< 0.001**$_{[1]}$	95	2.11	$0.15_{[1]}$
OX5195(2)BG1	79.5 ± 2.85	97 ± 1.21	16	10.8	**0.001**$_{[1]}$	100	0.52	$0.47_{[1]}$
OX5195(2)AY1	90.5 ± 2.07	88 ± 2.30	4	1.51	$0.22_{[1]}$	93	3.15	$0.08_{[1]}$
OX5195(2)BK1	91.5 ± 1.97	92 ± 1.92	3	0.97	$0.32_{[1]}$	97	0.74	$0.39_{[1]}$
OX5195(2)AR2	98 ± 0.99	95.5 ± 1.47	0	1.3	$0.25_{[1]}$	100	0.03	$0.86_{[1]}$
WT	95 ± 1.59							

SE: standard error. Df: degrees of freedom.

Table 5.6 Extent of repressibility of male sterility in OX5195(2) lines

Line	**Mean difference of hatch rates [NT – T] (%)**	**Significance**	
		X^2	$P_{[df]}$
OX5195(2)AY1	-2.5	0.33	$0.57_{[1]}$
OX5195(2)AR2	-2.5	0.99	$0.31_{[1]}$
OX5195(2)H1	-2	0.35	$0.55_{[1]}$
OX5195(2)AU1	0.0	0	$1_{[1]}$
OX5195(2)BK1	0.5	0.02	$0.90_{[1]}$
OX5195(2)AC2	1.0	0.17	$0.68_{[1]}$
OX5195(2)BG1	17.5	14.8	**< 0.001**$_{[1]}$
OX5195(2)AI1	26.5	19.4	**< 0.001**$_{[1]}$

Only weakly penetrant lines (5195(2)BG1 and AI1) were fully repressible.

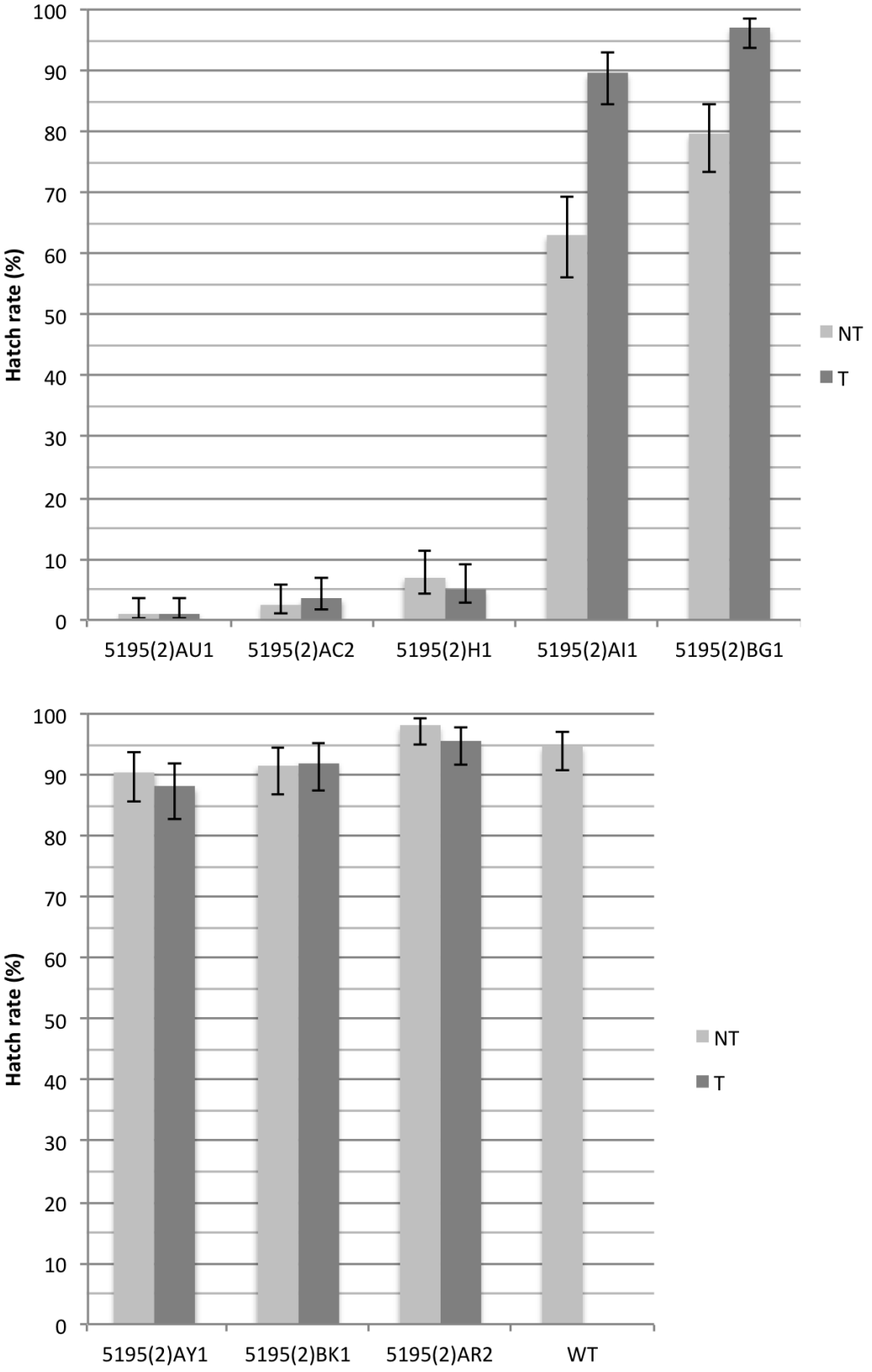


Figure 5.4. Highly sterile (penetrant) OX5195(2) lines demonstrate minimal tetracycline-mediated repressibility. Egg hatch assay demonstrates three phenotypic classes: impenetrant (> 90% viable: OX5195(2)AR2, BK1, AY1), semi-penetrant (60-80% viable: 5195(2)AI1 & BG1) and penetrant (< 10% viable: OX5195(2)AU1, AC2 & H1). Highly penetrant lines were not repressible but semi-penetrant lines were completely rescued by tetracycline. Error bars: 95% CI.

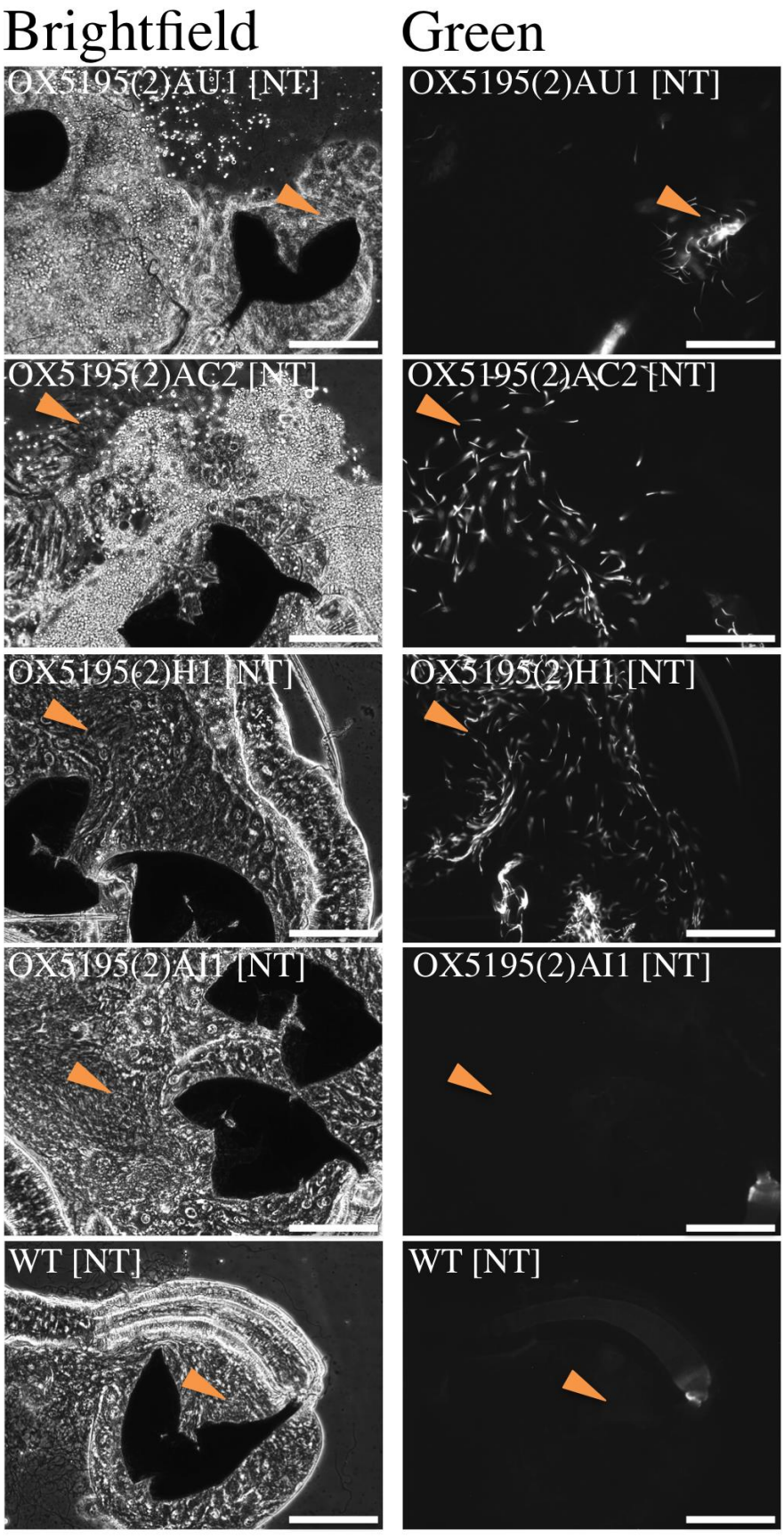


Figure 5.5 (Part 1). Fluorescent marker expression in sperm (Ccprot1-zsGreen) is correlated with irrepressible sterility in OX5195 lines (continued on next page). Fluorescence and phase contrast microscopy of dissected spermatechae of WT females mated to WT or OX5195(2) males, in order of penetrance (males were reared off-tet). Sperm (**shaded arrowhead**) are visible in all lines, but marked only in penetrant lines (OX5195(2)AU1, H1 & AC2). Very penetrant lines (OX5195(2)AU1 & AC2) transferred sperm in reduced quantity, relative to impenetrant lines and WT. Scale bars: 100 µm

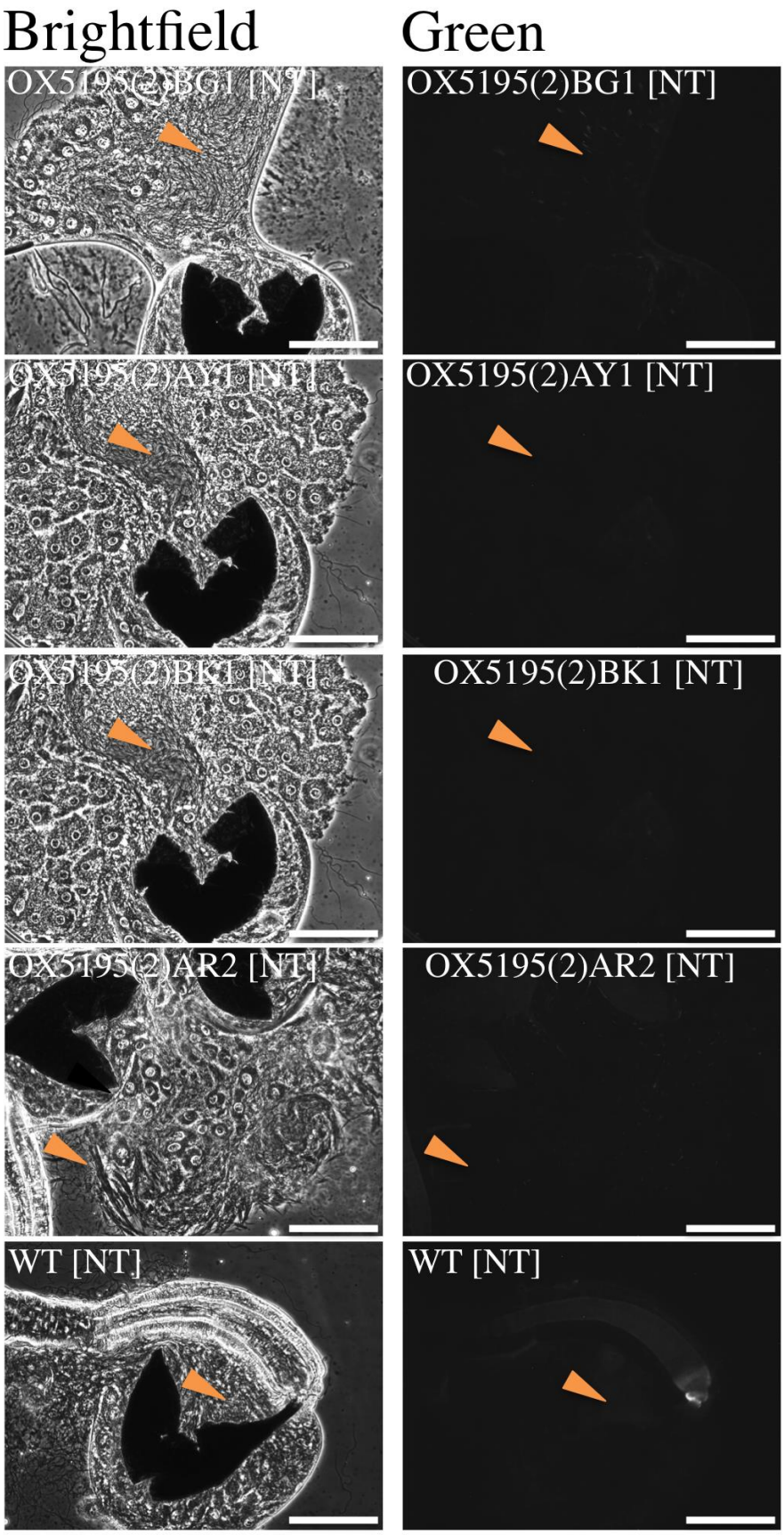


Figure 5.5 (Part 2). Fluorescent marker expression in sperm (Ccprot1-zsGreen) is correlated with irrepressible sterility in OX5195 lines. Fluorescence and phase contrast microscopy of dissected spermatechae of WT females mated to WT or OX5195(2) males, in order of penetrance (males were reared off-tet). Sperm (**shaded arrowhead**) are present in all lines, but marked only in penetrant lines (OX5195(2)AU1, H1 & AC2). Very penetrant lines (OX5195(2)AU1 & AC2) transferred sperm in reduced quantity, relative to impenetrant lines and WT. Scale bars: 100 μm

5.2.4 Non-repressible Ccprot1-full 5'UTR-FokI expression in males selects for weak insertions in male OX5195 G_0 individuals

We observed a significant female bias in transgenic OX5195(2) G_1 individuals (76% female, n=55, X^2=8.2; df=1, p=0.004). The most likely explanation is selection for X-linked insertions in male G_0 individuals (leading to female-specific inheritance of the expression construct in G_1 progeny). From 12 pools of G_0 individuals yielding transgenics (**Table 5.7**), male and female crosses were equally represented (six each). All lines derived from male G_0 crosses (n=5) were impenetrant or weakly penetrant. Five of six lines derived from female G_0 crosses were penetrant (all except OX5195(2)AR). Non-repressible expression would be expected to kill transformed germline cells in males but not females, because protamine-FokI should be exclusively expressed in males. Therefore, such insertions would be selected against. Although weak autosomal insertions are routinely observed, weak sex-linked insertions are more probable because essentially all of X and Y appear inhospitable for male germline expression. This is corroborated by published data (Parisi, 2003), (Koerich et al., 2008); and the observation that we have never recovered an X- or Y-linked insertion with penetrant expression in the male germline (OX5036 [**Section 3.7**], OX5150 [**Section 4.2.3**], OX5257 [**Section 5.5**]).

Three of five male G_0 lineages gave exclusively female transgenic progeny (OX5195(2)AY [n=7], BK [n=13] & BG [n=2]), consistent with X-linkage. The fourth (OX5195(2)AI) was a confirmed autosomal insertion and the fifth (OX5195(2)BA) was a confirmed Y-linked insertion. It would have been preferable to confirm this hypothesis experimentally by backcrossing heterozygous males of the impenetrant lines, but they had been discarded by the time the relationship was discovered. Consequently, we calculated probability of X-linkage for the male crosses deriving only females, assuming no secondary autosomal or Y insertions. This is represented by the inverse probability of randomly observing that particular sex distribution: $1-(1/2^n)$, where n is number of females present.

Table 5.7: Non-repressible expression of Ccprot1-full 5'UTR-FokI (OX5195) selects for weak insertions in G_0 microinjected males

G_0 pool	**G_0 sex**	**G_1 progeny**			**Male sterility phenotype**		**Probability of X-linkage**
		♂	♀	Total	Penetrance	Repressibility	
OX5195(2)AY	♂	**0**	7	7	None		99.2%
OX5195(2)BG	♂	**0**	2	2	Weak	Full	75%
OX5195(2)BK	♂	**0**	13	13	None		> 99.9%
OX5195(2)AI	♂	2	2	4	Weak	Full	Known autosomal insertion
OX5195(2)BA	♂	2	**0**	2	None		Known Y-linked insertion
OX5195(2)H	♀	0	3	3	Strong	None	
OX5195(2)P	♀	1	1	2	Strong	None	
OX5195(2)AC	♀	1	3	4	Strong	None	
OX5195(2)AR	♀	**0**	2	2	None		
OX5195(2)AU	♀	4	9	13	Strong	None	
OX5195(2)AW	♀	2	**0**	2	Strong	None	

5.2.5 Transcription of Ccprot1-full 5'UTR-FokI is partially repressed in OX5195(2)AU1, but this does not improve embryonic viability

We investigated Ccprot1-FokI expression in testes by qPCR analysis of the most penetrant line (OX5195(2)AU1), and a relatively impenetrant line that demonstrated normal hatch rate in the presence of tetracycline (OX5195(2)AI1), to assess whether tetracycline had any repressive effect on the male sterility effector. Triplicated samples were five pairs of testes from OX5195(2) heterozygotes or WT (aged 7 days). Dissections, RNA extraction and qRT-PCR were as previously described (**Section 2.1.3.4**). Expression levels were normalised to a housekeeping gene (*Cc-Rp17S*) and subsequently to the normalised mean expression level of tetracycline-reared OX5195(2)AI1 males (expected to indicate a baseline level of expression). Results are summarised in **Table 5.8** and **Figure 5.6**. Partial repression was observed for the highly penetrant line OX5195(2)AU1[T] (71% repressed, relative to baseline). As the progeny of crosses of OX5195(2)AU1[NT] and [T] males were equally inviable, it appears that hundred-fold induction of Ccprot1-FokI is sufficient to reduce hatch rate to 1%. The results indicated considerable variation in expression between biological replicates (large standard error). Consequently, a detailed statistical analysis would likely be misleading. Future analysis would be improved by performing five biological replicates, each consisting of 15 pairs of testes, with an averaged measurement from three technical replicates of each biological replicate. It was not necessary to repeat these results, as we demonstrated in a parallel analysis that shortening the Ccprot1 5'UTR was sufficient to engineer almost complete repression (**Section 5.4**). Therefore, the

relative expression levels of Ccprot1-full 5'UTR-FokI in these lines were no longer of interest.

Table 5.8 Normalised Ccprot1-FokI transcription levels in OX5195(2) testes

Line	**Fold change ($2^{-\Delta\Delta Ct}$)**			
	NT		T	
	Mean	Range (Mean ± SE)	Mean	Range (Mean ± SE)
OX5195(2)AU1	348.1	226.8-534.2	101.8	87.1-119.0
OX5195(2)AI1	3.1	0.4-22.9	1	0.3-3.6
WT	0.2	0.1-0.6	0.5	0.2-1.0

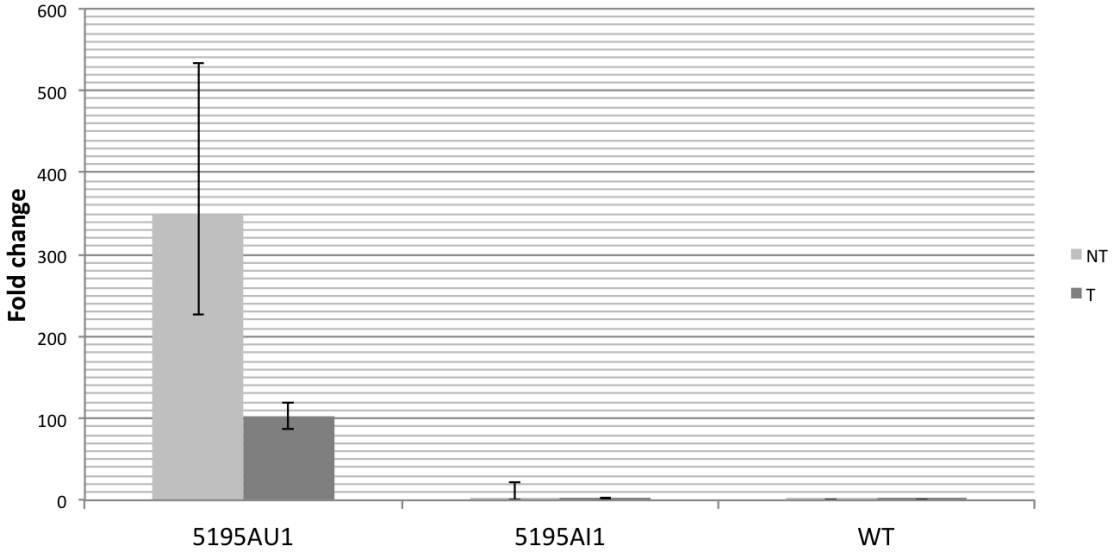


Figure 5.6. qRT-PCR analysis of Ccprot1-full 5'UTR-FokI expression in OX5195(2)AU1 and AI1. All measurements were expressed relative to the calibrator sample (OX5195(2)AI1 [T]). Expression of Ccprot1-full 5'UTR-FokI in OX5195(2)AU1 was strongly induced in off-tet reared males [NT] and considerably repressed in tet-reared males [T], but not sufficiently to reverse sterility. The impenetrant line OX5195(2)AI1 demonstrated minimal induction off-tet. Error bars: log-transformed standard error.

5.2.6. An embryonic staining assay to visualise the effect of paternal expression of protamine-FokI

We considered that the paternal effect of Ccprot1-FokI expression on embryonic development could be visualised by nuclear staining, providing context for the developmental effects of penetrant, but incompletely repressed Ccprot1-full 5'UTR-FokI. This was intended as a preliminary experiment to determine the extent to which embryonic development was arrested by protamine-FokI expression, and to optimise testing for commercially relevant, fully repressible male sterility systems. Lines of varying penetrance were tested to maximise informational output for assay development: OX5195(2)AU1 [fully penetrant, non-repressible]; OX5195(2)AI1 [semi-penetrant, fully repressible]; and OX5195(2)AR2 [completely impenetrant]). The F_0 cross was heterozygous transgenic females [T] (n=10) to WT males [NT] (n=5). F_1 progeny were filtered on- and off-tet. Heterozygous males (n=25) of each treatment (NT or T) were crossed to WT females [NT] (n=50). Controls were equivalent crosses with WT males (NT and T).

Eggs were collected in four hour cycles, fixed and stained. Two timepoints were assessed: 0-4 hours (fixed immediately on day 9) and 20-24 hours (collected on day 7, and left to develop for 20 hours). We imaged and phenotypically assessed at least 10 individuals per group. Embryos from the 0-4 hour collection were staged as developing or not developing (several nuclei visible, or only the first nucleus visible); and from the 20-24 hour collection as morphologically normal (N), morphologically aberrant (A), or no visible development (ND). For statistical analysis, data was divided by timepoint and correction-free chi-square testing was performed on the number of individuals from each category, relative to the appropriate WT control (NT or T). Repression was calculated by chi-square testing the number of individuals from each phenotypic category, for the [NT] and [T] observations for each line. We hypothesised the stage at which embryonic development would arrest, based on the observed hatch rate of progeny of off-tet and on-tet reared males, from the prior section (**Table 5.9**).

Table 5.9: Expected embryonic developmental phenotypes

Line	Mean hatch rate (%)		Embryonic developmental phenotype	
			Expected	**Observed**
	NT	**T**		
5195(2)AU1	1.0	1.0	Early developmental arrest in the majority of embryos, with no effect of tetracycline.	Early and late development were affected, but embryos were able to proceed to a much later stage than expected. Tetracycline had no obvious effect.
5195(2)AI1	63.0	89.5	Developmental arrest in a proportion of embryos, when reared off-tet. Normal development when reared on-tet.	No effects on early development detected. Mild effects on late development were indicated (off-tet and on-tet), but were not statistically significant. Tetracycline appeared to rescue development, as expected.
5195(2)AR2	98.0	95.5	Normal development	
WT	95			

Early embryonic development (0-4 hours) was affected by paternal expression of Ccprot1-full 5'UTR-FokI for OX5195(2)AU1; a significantly higher rate of non-development was observed both off-tet (n=12, X^2 =26.0, p < $0.001_{[1]}$) and on-tet (n=10, X^2 =6.84, p= $0.009_{[1]}$), relative to WT controls (**Table 5.10**). Neither OX5195(2)AI1 nor OX5195(2)AR2 differed significantly from controls. This indicated that the basis of reduced hatch rate in OX5195(2)AI1 might be later-acting; that the scoring method did not detect differences at this stage (binary scoring of embryos as developed or non-developed, might not reveal minor effects); or that the sample size was too small to detect a minor effect on viability. Tetracycline-mediated developmental differences (repression) were not detected for any line, except for a small but marginally significant result for WT control (n=29 [NT=14, T=15], X^2 =4.33, p=$0.04_{[1]}$). This may indicate a genuine, tetracycline-mediated developmental delay; the impenetrant line OX5195(2)AR had a similar result (marginally non-significant, n=27 [NT=11, T=16], X^2 =3.23, p=$0.07_{[1]}$). However, the effect of small sample size and the possibility that a proportion of eggs were recently laid rather than undeveloped cannot be excluded. Hence, future assays would be improved by applying a larger sample size and a smaller window of collection (10-30 minute cycles rather than 4 hours).

Table 5.10: Early embryonic development is arrested in OX5195(2)AU

Line	Embryo development				Significance testing					
	NT		**T**		**NT**		**T**		**Repression**	
	Developed	Not	Developed	Not	X^2	$P_{[df]}$	X^2	$P_{[df]}$	X^2	$P_{[df]}$
5195(2)AU1	0	12	2	8	**26.0**	**<0.001[1]**	**6.84**	**0.009[1]**	2.64	$0.10_{[1]}$
5195(2)AI1	11	2	12	3	2.33	$0.13_{[1]}$	0.19	$0.67_{[1]}$	0.10	$0.75_{[1]}$
5195(2)AR2	11	0	12	4	0.01	$0.95_{[1]}$	0.01	$0.92_{[1]}$	3.23	$0.07_{[1]}$
WT	14	0	11	4					**4.33**	**0.04[1]**

Correction-free chi-square testing was performed on the number of individuals from each phenotypic category, relative to the appropriate WT control (NT or T). Repression was calculated by chi-square testing the number of individuals from each phenotypic category, between the [NT] and [T] observations for each line.

The extent of late embryonic development (20-24 hours) was not as expected (**Table 5.11, Figure 5.7**). Despite early developmental delay (0-4 hours) in progeny of OX5195(2)AU1[NT] males, morphologically normal embryos were frequently observed (46%). It was not expected that development would proceed to such a late stage, given that embryos in the early assay (0-4 hours) did not generally appear to exhibit cellular divisions. Nonetheless, viability was significantly reduced relative to WT controls, both off-tet (n=24, X^2 =11.1, $p=0.004_{[1]}$) and on-tet (n=20, X^2 =11.7, $p=0.003_{[1]}$). There was no significant difference in development for embryos of the semi-penetrant line OX5195(2)AI1, relative to WT controls (off-tet or on-tet). This was unexpected, as progeny of OX5195(2)AI1[NT] males demonstrated reduced hatch rate. Again, it is possible that the sample size was too small to detect a minor effect, or that the developmental arrest that underpinned a reduction in hatch rate tended to occur at a later stage than that assessed. As expected, the impenetrant line OX5195(2)AR2 did not differ significantly from controls. Tetracycline-mediated repression (improved viability in progeny of on-tet reared males) was only detected for OX5195(2)AI1 (n=40 [NT=20, T=20], X^2 =10.0, $p=0.007_{[1]}$), as expected from the observation that tetracycline rescued embryonic viability, in the prior hatch assay. No significant effect of tetracycline on WT development was observed (n=40 [NT=20, T=20], X^2 =1.56, $p=0.046_{[1]}$). This indicates that the differences in viability between WT[NT] and WT[T] in the prior assay (0-4 hours), were likely to be artefactual.

Table 5.11: Late embryonic development is arrested in OX5195(2)AU and rescued by tetracycline in OX5195(2)AI

Line	**Embryo development**						**Significance testing**					
	NT			**T**			**NT**		**T**		**Repression**	
	ND	A	N	ND	A	N	X^2	$P_{[df]}$	X^2	$P_{[df]}$	X^2	$P_{[df]}$
5195(2)AU1	9	4	11	5	10	5	**11.1**	**$0.004_{[1]}$**	11.7	**$0.003_{[1]}$**	5.65	$0.06_{[1]}$
5195(2)AI1	0	8	12	0	0	20	4.80	$0.091_{[1]}$	5.71	$0.06_{[1]}$	**10.0**	**$0.007_{[1]}$**
5195(2)AR2	2	0	18	0	0	20	4.0	$0.14_{[1]}$	5.71	$0.06_{[1]}$	2.11	$0.35_{[1]}$
WT	0	2	18	0	5	15					1.56	$0.46_{[1]}$

The assessment of statistical significance was conducted as described in the above table. Abbreviations: ND (no development), A (morphologically aberrant); or N (morphologically normal).

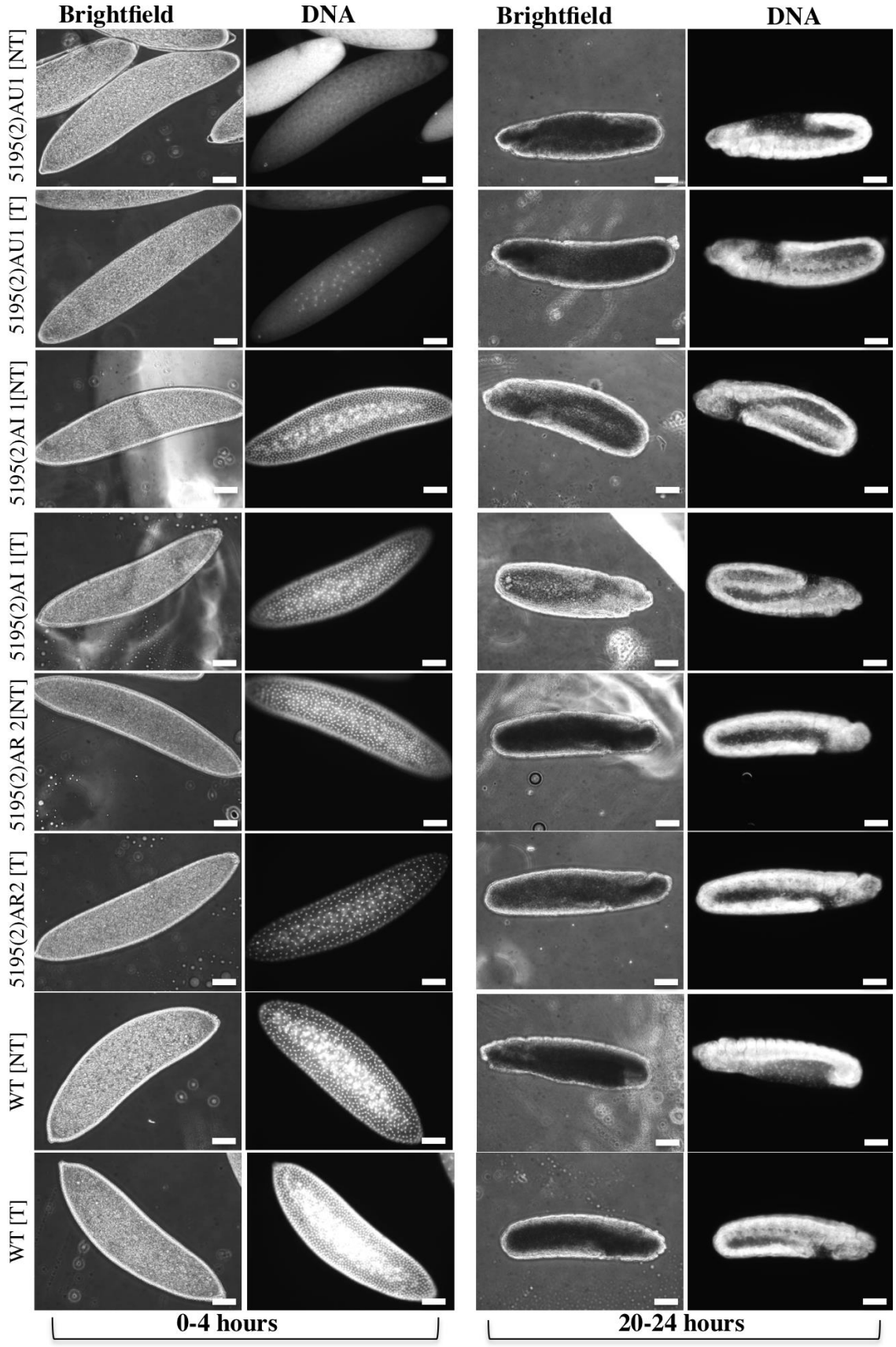


Fig. 5.7. Paternal expression of Ccprot1-full 5'UTR-FokI blocks embryonic development. Nuclear stained embryos from crosses of WT females to OX5195 or WT males. Lines of varying penetrance for sterility were tested. OX5195(2)AU1 (penetrant, non-repressible) embryos have tetracycline-independent, minimal early development (0-4 hours) but most demonstrate later development than expected. OX5195(2)AI1 (semi-penetrant, fully repressed) was minimally affected. OX5195(2)AR2 (impenetrant) was unaffected. Scale bars: 100 μm.

The extent of post-blastodermal development in OX5195(2)AU was surprising, as we did not expect that a genome sufficiently damaged to block larval hatching would mediate organogenesis. It is likely that the maternal haploid genome is minimally affected (by degradation of protamine-FokI or titration by binding to paternal DNA) and hence can maintain partial development. Interestingly, a variety of mutants are known in *D. melanogaster*, wherein the paternal genome is prevented from mediating embryonic development. These may be maternal-effect (*maternal haploid* and *sésame*) or paternal-effect (*sneaky* and *misfire*). In homozygous *maternal haploid* (*mh*) mutants, the paternal chromatids do not form functional centrosomes. They are excluded in subsequent divisions, and a substantial proportion of embryos develop as haploid gynogenetic embryos from the maternal genome. About 22% reach a late stage of embryonic development, with cuticular deposition; however, none hatch (Loppin et al., 2001). *sésame* is required for the decondensation of sperm chromatin; in mutants, the paternal DNA is excluded from subsequent divisions. Although hatching is not observed, the majority (72%) proceed to late embryonic development and form a cuticle; about 7% arrest in early development (Loppin et al., 2000). It is interesting to note that we observed organogenesis in a similar proportion, 15/24 (63%) of OX5195(2)AU1[NT] embryos at 20-24 hours. However, the frequency of early arrest (0-4 hours) was greater (no development in 9/24 [37%] of embryos). *Sneaky* and *misfire* mutants appear to be defective in the breakdown of the sperm plasma membrane upon entry into the egg, and the majority do not develop whatsoever. Interestingly, about 1% of embryos hatch, similar to the rate reported for OX5195(2)AU1, though the mode of action is clearly different. This is because OX5195(2)AU1 embryos frequently arrest in later development, rather than the first division (Fitch and Wakimoto, 1998), (Ohsako et al., 2003).

Although it is clear that Ccprot1-FokI expression induces double-stranded breaks sufficiently to prevent development to the larval stage, this is not sufficient to mediate early arrest, in the majority of instances. Aneuploidy is frequently observed in *mh* embryos, which nonetheless frequently develop as haploid embryos (Loppin et al., 2001). From this observation, even severe paternal chromosomal aberrations resulting in aneuploidy, will not arrest embryonic development in every instance. This indicates that should an earlier arresting phenotype be desirable, that fragmentation of the paternal genome is not sufficient. Though this extent of late development was not

expected, it is not inherently problematic, because only 1% of embryos from crosses of WT females to heterozygous OX5195(2)AU1 transgenic males hatched. Viable adult progeny were never observed in crosses of heterozygous transgenic males (reared off-tet or on-tet), suggesting that the 1% of individuals that hatched died at a later stage (probably larval, as pupae were never observed). Of course, this would need to be confirmed by a replicated pupation and eclosion assay, for the observation to be fully valid.

From these results, it was not possible to determine if the undeveloped embryos resulted from an early paternal effect lethal phenotype; or if they were instead unfertilised (potentially as a consequence of deficient sperm transfer). To confirm the mode of sterility in these instances, it would be theoretically possible to determine if the male haploid nucleus was present using sensitive PCR techniques such as digital PCR (Ottesen et al., 2006). Alternatively, it would be interesting to determine if the whole sperm tail enters the egg as it does in *D. melanogaster*, as this would facilitate staining the egg to visualise the sperm tail (Karr, 1991). Fusions of GFP to don-juan (dj), a sperm tail protein have been developed in *D. melanogaster* (Santel et al., 1997), though we were unable to find a homologue in Medfly (NCBI, 2016). Alternatively, it may be possible to visualise the male haploid nucleus by Ccprot1-zsGreen (the fluorescent sperm marker) localisation. We did not image the embryos under the green filter; it would be interesting to determine if this was visible.

Improvements for future application of this assay are suggested. The sample size should be increased. Scoring more than 100 embryos would reduce probability of artefactual effects, which was indicated in two instances. First, progeny of WT[NT] males developed marginally better than WT[T] equivalent (early development: 0-4 hours). Second, embryos of the semi-penetrant line OX5195(2)AI1 were marginally more viable on-tet than the WT control (though this result was not statistically significant). No reasonable biological basis could be ascribed to these findings, indicating that they are artefactual. Furthermore, shorter collection cycles should be performed. Across four hours, development differs considerably and may reduce scoring accuracy. This was pertinent for early scoring (0-4 hours), where certain eggs were possibly recently laid. Absence of undeveloped eggs in the WT [NT] control indicates this was not frequent. However, performing 10-30 minute collections and ageing to the desired stage would eliminate this possibility; and narrow the range of

development between embryos. It would also be useful assess later stages of development (> 24 hours). The penetrant line OX5195(2)AU1 demonstrates a 1% hatch rate; it would be interesting to investigate later embryonic development, which is clearly not arrested in every instance. Finally, the accuracy of scoring could be improved with proper staging. Early embryos (0-4 hours) were classed as developed/undeveloped, without respect to the number of divisions present. Late embryos (20-24 hours) were scored as morphologically normal, morphologically aberrant, or no visible development. Therefore, these classifications spanned very broad stages of embryonic development, and are vague in comparison with the stage classifications possible by other models, such as the 17-stage model widely used in *D. melanogaster* (Campos-Ortega and Hartenstein, 1997). It would also be useful to pair the assessments of embryonic development, with quantitative molecular techniques. A molecular assay, such as qPCR estimation of genomic copy number, might enhance sensitivity. Comparable methods have been applied to quantify the number of viral genomes within insect tissue to determine the likelihood of infectiousness (Walker et al., 2011), (Bian et al., 2013).

5.2.7 Modification of the Ccprot1-FokI and Ccprot2-FokI male sterility effectors to engineer repressibility

Next, we considered the potential factors mediating non-repressibility in OX5195 and revised construct design, to develop a tetracycline-repressible variant. First, we considered if repressibility might be insertion sensitive, which has been observed for tetracycline-repressible transgenic systems developed at Oxitec. An *A. albopictus* flightless line was non-repressible in about 20% of lines (Labbé et al., 2012). However, failure to isolate a single penetrant and repressible candidate from a panel of 11 lines, indicates a non-repressible expression pattern that cannot be attenuated by insertion. Next, we considered if the dosage of tetracycline was inadequate to repress transcription of tetO-Ccprot1 full 5'UTR-FokI. Full repression of sterility in semi-penetrant lines (OX5195(2)AI1 & BG1) indicated that the system was partially responsive to tetracycline. Similarly, q-RT-PCR analysis indicated partial repression of Ccprot1-full 5'UTR-FokI expression in the penetrant but non-repressible line OX5195(2)AU1, though to an insufficient extent to restore fertility. Possibly, higher dosage or stronger analogues (eg. doxycycline) could promote repression (Curtis et al., 2015). Recently, an expression system in *D. suzukii* with a similar male sterility effector (tetO21-hsp70-Dmprot1-FokI) and transcriptional activator (Dmβ2T-Dmhsp83-tTAV)

was sterile but not repressible. A five-fold excess of tetracycline, relative to the standard Medfly rearing concentration (500 ng/µl), did not attenuate this effect (Megas et al., unpublished data). Furthermore, the same dosage of tetracycline applied in the OX5195(2) male sterility experiments (100 ng/µl), provided full repression of sterility in previously developed systems (OX4353/4718; **Figure 3.2** and **Figure 3.5**). As the β2T-tTAV-tetO21-Dmhsp70 minipromoter fragment driving Dmprot2-chimeric-FokI was essentially identical (the Dmhsp70 5'UTR was shortened from 89 to 21 bp), it appeared likely that sequences within Ccprot1-FokI reduced repressive capacity.

The interaction of tTAV with tetO21-minimal promoter is required for transcriptional activation, and this is displaced by tetracycline (Gossen, 1992). Consequently, if a downstream promoter element is present (for example in the 5'UTR), it could potentially act independently of the tet-repressible system. The observed tetracycline-mediated, three-fold reduction in OX5195(2)AU1 transcript was consistent with this model; as was the indication of potential downstream transcriptional start sites (TSS) by 5'RACE on Ccprot1-zsGreen (**Section 4.4.1**). In *D. melanogaster*, sequences within the 5'UTR can enhance transcription in the male germline (Kempe et al., 1993). Therefore, we reasoned that shortened Ccprot1 or Ccprot2 5'UTRs were likely to provide repressible FokI expression. We attempted to engineer repressibility by shortening the Ccprot1 and Ccprot2 5'UTRs to a more minimal sequence (the furthest downstream TSS, as indicated by 5'RACE or NCBI high-throughput transcript sequencing data). This theory was tested by generating constructs applying truncated 5'UTR sequences (**Figure 5.8**): OX5242 (Ccprot1-short 5'UTR-FokI) and OX5241 (Ccprot2-short 5'UTR-FokI).

A

```
Ccprot1     [49 bp]…AATCGTACAAATTCTAGTGAAAACGATATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#10               AATCGTACAAATTCTAGTGAAAACGATATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#8                      AAATTCTAGTGAAAACGATATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#2,3                                 ATACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#1,9                                 TACATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#11                                    ATAGTTATAAAAAATTTGTTG…[106 bp]…ATG
#4,7                                   AGTTATAAAAAATTTGTTG…[106 bp]…ATG
#5                                                    G…[106 bp]…ATG

Ccprot2                          [25 bp]…AGACAATTGAAAATTTCCAGTCAT…[114 bp]…ATG
#3                                     AGACAATTGAAAATTTCCAGTCAT…[114 bp]…ATG
#6,7                                         GAAAATTTCCAGTCAT…[114 bp]…ATG
#1,8                                                  AT…[114 bp]…ATG
#2,4,5                                                 T…[114 bp]…ATG
```

B

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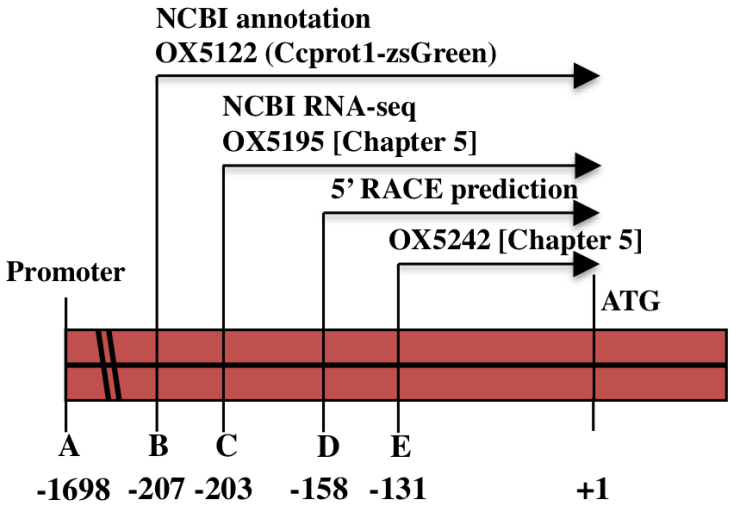
Ccprot1 (Annotation)  TGTATATCATTTTAAAACCATTTGCGCAAATACATTTGAAAATGTTTTCA…[157 bp]…ATG
Ccprot1 (RNA-seq)         TATCATTTTAAAACCATTTGCGCAAATACATTTGAAAATGTTTTCA…[157 bp]…ATG
Ccprot1 (5′ RACE)                                                  A…[157 bp]…ATG

Ccprot2 (Annotation)  …[26 bp]…AGACAATTGAAAATTTCCAGTCATTCGTTTTACATTTTTTTT…[97 bp]…ATG
Ccprot2 (RACE)              AGACAATTGAAAATTTCCAGTCATTCGTTTTACATTTTTTTT…[97 bp]…ATG
Ccprot2 (RNA-seq)                                                ATTTTTTTT…[97 bp]…ATG
```

C



D

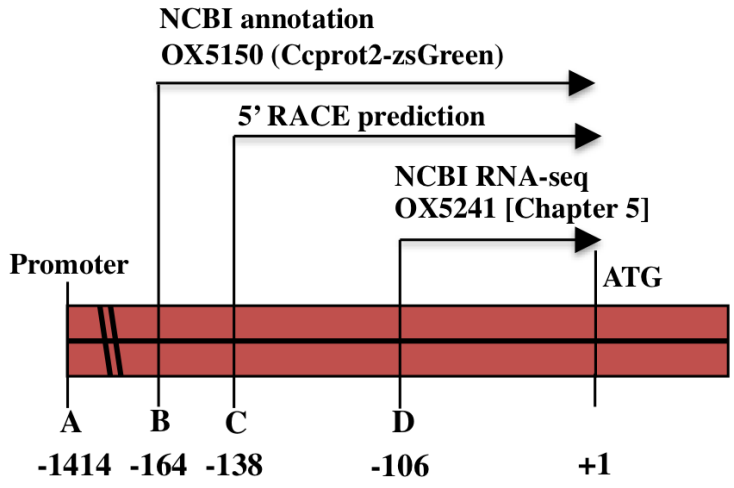


Figure 5.8. Truncation of Ccprot1 and Ccprot2 5'UTRs to remove cryptic elements promoting transcription independently of tetracycline (continued on next page). **(A-B)** 5' RACE indicates multiple transcriptional start sites (TSS) within the Ccprot1 and Ccprot2 5'UTRs, consistent with secondary promoters that could mediate non-repressible sterility. **(A)** Seven potential TSS for Ccprot1 and four for Ccprot2 were indicated. **(B)** Alignments of potential TSS: annotation from NCBI; NCBI RNA-seq data (furthest upstream sequence); and 5' RACE (furthest upstream sequence). (**C-D**) Positions of the potential TSS for Ccprot1 (**C**) and Ccprot2 (**D**), relative to the upstream promoter termini and first codons. Sequences applied in OX5241 (Ccprot2) and OX5242 (Ccprot1) for sterility (as Dmhsp70-Ccprot-FokI chimerae) are indicated.

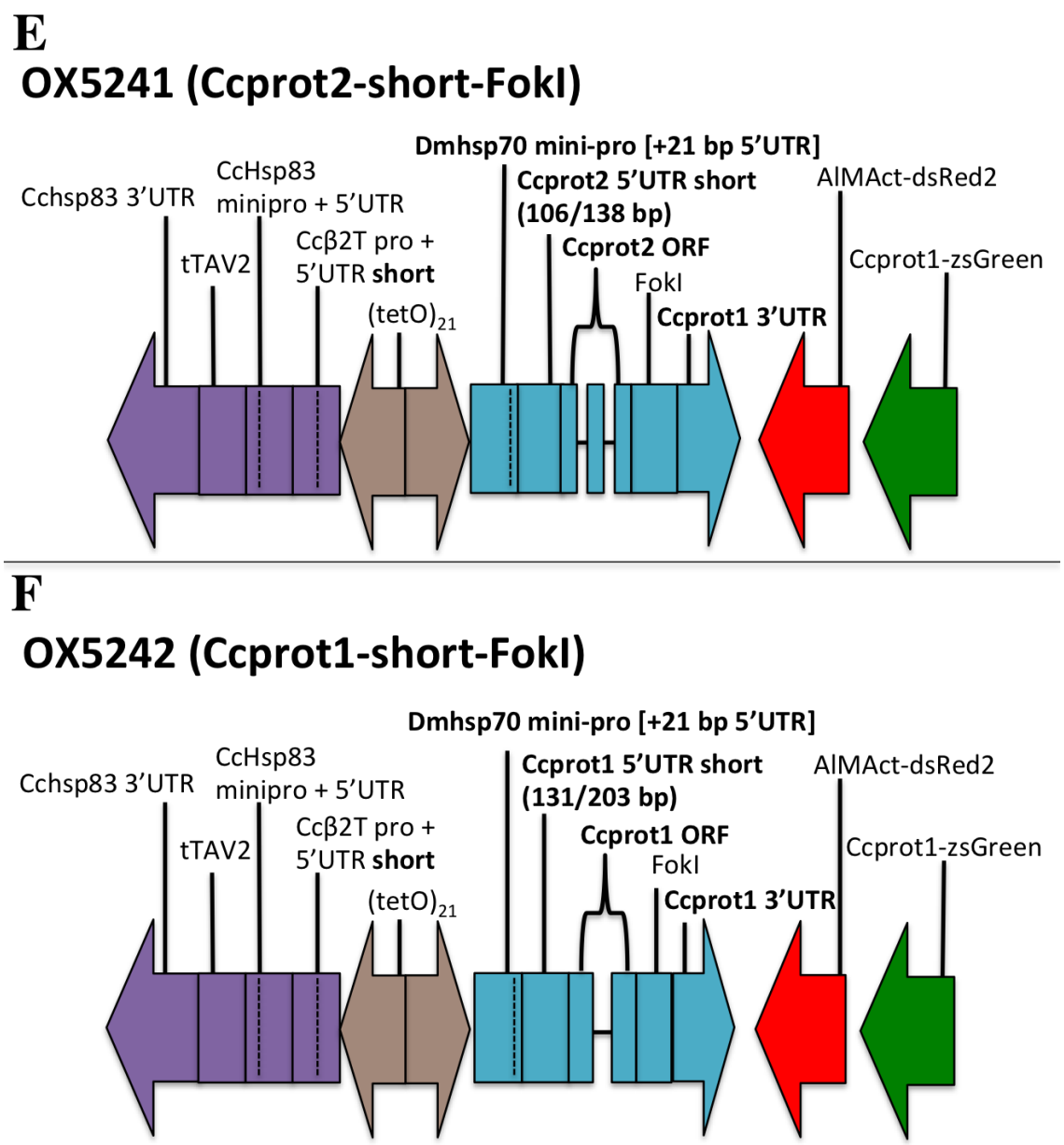


Figure 5.8. Truncation of Ccprot1 and Ccprot2 5'UTRs to remove cryptic elements promoting transcription independently of tetracycline. (E-F) Expression construct diagrams for OX5241 **(E)** and OX5242 **(F)**. Dashes indicate promoter-5'UTR boundary. Note: piggyBac ends omitted from these four-ended vectors (full diagrams shown in Figure 2.2).

5.3 Heterozygous OX5241 (Ccprot2-short 5'UTR-FokI) males are irrepressibly sterile in penetrant insertions

5.3.1 Establishment of OX5241 transgenic lines

Microinjection (**Table 5.12**), backcrossing to WT (**Table 5.13**), screening for transient expression of the transformation marker and Mendelian assessment of transgenic lines were largely as described for OX5195. Buffered mixes contained tetracycline (100 ng/µl), OX5241 (600 ng/µl), and *piggyBac* DNA helper OX3022 and RNA helper OX3081 (300 ng/µl each). Transient AlMAct-dsRed2 expression was

observed in G_0 pupae, indicating successful microinjection. Adult survival (16%) was slightly reduced compared to typical results for injection of Medfly (25%) (Gregory et al., 2016). Transformation efficiency was low (1.2%), but typical for a very large construct (OX5195/OX5242/5257 microinjections [**Sections 5.2, 5.4, 5.5**]).

Table 5.12 Microinjection logistics for OX5241

Embryos	Larvae	Pupae	Adults	Lines
2715	782 (29%)	492 (18%)	429 (16%)	5 (1.2%)

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (transgenic lines/G_0 adults crossed).

Table 5.13 G0 backcrosses to establish OX5241 lines

OX5241A	10 ♂ x 30 WT ♀	OX5241B	6 ♂ x 30 WT ♀	OX5241C	10 ♀ x 10 WT ♂
OX5241D	10 ♂ x 30 WT ♀	OX5241E	9 ♂ x 30 WT ♀	**OX5241F**	**8 ♀ x 8 WT ♂**
OX5241G	8 ♀ x 8 WT ♂	OX5241H	8 ♂ x 30 WT ♀	OX5241I	7 ♂ x 30 WT ♀
OX5241J	7 ♀ x 7 WT ♂	OX5241K	7 ♀ x 7 WT ♂	OX5241L	20 ♀ x 10 WT ♂
OX5241M	10 ♂ x 30 WT ♀	OX5241N	10 ♂ x 30 WT ♀	OX5241O	4 ♂ x 30 WT ♀
OX5241P	10 ♂ x 30 WT ♀	OX5241Q	5 ♂ x 30 WT ♀	OX5241R	10 ♀ x 10 WT ♂
OX5241S	19 ♀ x 10 WT ♂	OX5241T	9 ♂ x 30 WT ♀	OX5241U	10 ♂ x 30 WT ♀
OX5241V	7 ♂ x 30 WT ♀	OX5241W	20 ♀ x 10 WT ♂	OX5241X	11 ♂ x 30 WT ♀
OX5241Y	11 ♂ x 30 WT ♀	OX5241Z	11 ♂ x 30 WT ♀	**OX5241AA**	**10 ♂ x 30 WT ♀**
OX5241AB	**22 ♀ x 10 WT ♂**	**OX5241AC**	**20 ♀ x 10 WT ♂**	OX5241AD	19 ♀ x 10 WT ♂
OX5241AE	23 ♀ x 10 WT ♂	OX5241AF	13 ♂ x 30 WT ♀	OX5241AG	13 ♂ x 30 WT ♀
OX5241AH	13 ♂ x 30 WT ♀	OX5241AI	14 ♂ x 30 WT ♀	OX5241AJ	8 ♂ x 30 WT ♀
OX5241AK	**17 ♀ x 10 WT ♂**				

Underlined crosses gave rise to transgenic lines.

5.3.2 OX5241 transgenic G_1 male individuals are frequently infertile

Five transgenic pools were isolated: OX5241F, AA, AB, AC, & AK. As a preliminary test of repressibility, males and females from each pool were independently crossed to WT. All rearing and crosses were performed in the presence of tetracycline. Eggs were filtered to diet (day 7) and qualitatively scored for development (day 12). Spermathecae of females mated to transgenic males were dissected and scored for marked sperm (day 7), an indicator of penetrance (at Oxitec). A detailed investigation of fluorescent sperm marking was not pursued because no lines provided a commercially applicable phenotype (penetrant and repressible sterility).

Infertility of male G_1 individuals was observed in three of the four pools where males were present (OX5241AA, AB & AK). Females in all four of the four pools where females were present (OX5241F, AB, AC & AK) were fertile **(Table 5.14**). Males were absent in OX5241AC and females absent in OX5241AA. Interestingly, OX5241AB1 (♂) appeared semi-fertile, when reared on tetracycline. Transgenic males transferred sperm to WT females on mating, in all cases. Fluorescent marking in sperm nuclei was observed in OX5241AA1, AB1 & AK1 (infertile individuals); but not OX5241F3 (fertile individual). Therefore, as with OX5195 (Ccprot1-full 5'UTR-FokI), penetrance of the male sterility phenotype and sperm marking appeared to be correlated (**Figure 5.9**). We performed further analysis in the G_2 generation for lines OX5241F3, AB1, AC2, and AK3 (the male infertile OX5241AA1 insertion was lost). All lines except OX5241F3 were established from females (because the males were infertile). Given the low transformation efficiency of the construct, it was assumed that males and female transgenic individuals of the same pool had the same transgenic insertion, and therefore hypothesised that all penetrant lines would be confirmed non-repressible.

Table 5.14: Fertility of OX5241 transgenic G_1 individuals

G_1 individual	**Sex**	**Fertile**	**Sperm marking**	**Apparent phenotype**
OX5241F3	♂	Yes	No	Impenetrant male sterility, no fluorescent sperm marking
OX5241AB1	♂	Partially	Yes	Semi-penetrant male sterility with fluorescent sperm marking
OX5241AA1	♂	No	Yes	Penetrant but non-repressible male sterility with fluorescent sperm marking
OX5241AK1	♂	No	Yes	
OX5241F1	♀	Yes		
OX5241AB3	♀	Yes		
OX5241AC2	♀	Yes		
OX5241AK3	♀	Yes		

Boldface crosses were used to establish permanent transgenic lines for further phenotypic analysis.

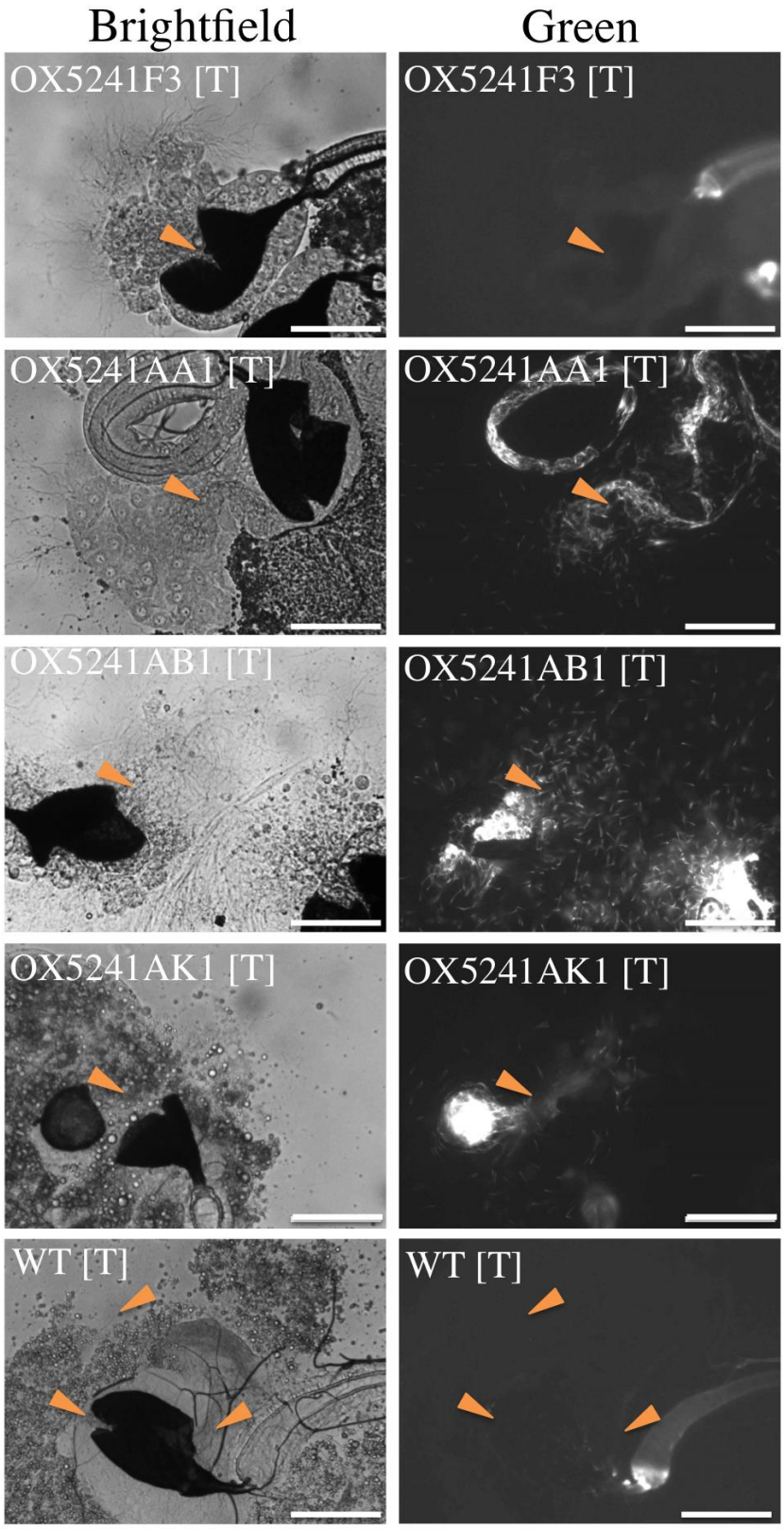


Figure 5.9. Fluorescent marker expression in sperm (Ccprot1-zsGreen) transferred from G_1 transgenic individuals of OX5241 (Ccprot2-short 5'UTR-FokI) is correlated with irrepressible sterility. OX5241 G_1 transgenic males (tet-reared) were mated to WT females; spermatheace were dissected. Sperm (shaded arrowheads) was transferred in all instances, and marked in all individuals except OX5241F3. OX5241AA1 & AK1 were infertile, and OX5241F3 fertile, as expected. Interestingly, OX5241AA1 was semi-fertile this generation, but appeared completely infertile when its progeny were assessed by egg hatch assay the subsequent generation. Scale bars: 100 µm.

5.3.3 OX5241 lines with penetrant male sterility are not repressible

Mendelian properties of each insertion were assessed as previously described (**Table 5.15).** OX5241F3 was a single X-linked insertion; OX5241AB1 was autosomal; the others were not determined (sex-linkage is not evident from crosses of transgenic females). The penetrance and repressibility of the male sterility phenotype were assessed by egg hatch assay, essentially as described for OX5195(2) [**Section 5.2.3**]. Five heterozygous males of each treatment (non-tet or tet reared) were independently crossed to ten non-tet reared WT females. Controls were equivalent crosses with WT males and WT females. 24 hour egg collections (n=100 eggs) were performed on day 5, 6 and 7 post-cross. Statistical assessment was as described for OX5195(2).

Table 5.15 Mendelian analysis of established OX5241 transgenic lines

Line	Sex	Copy number		Sex-linkage		Summary	
		Transgenic (%)	n	Ratio (M/F)	n	Copies	Location
OX5241F3	♂	47	437	Female only	86	1	X
OX5241AB1	♂	48	83	2.5	28	1	Autosome
OX5241AC2	♀	47	199	1	81	1	Unknown
OX5241AK3	♀	52	113	1.4	60	1	Unknown

All transgenic insertions appeared to be single copy. OX5241F3 was X-linked and OX5241AB1 was autosomal. It was not possible to assess sex-linkage of 5241AC2 and AK3 because insertions were male infertile. "n": number of individuals assessed.

The assay results generally recapitulated the phenotypes indicated by the presence of infertile males in the G_1 transgenic crosses (**Tables 5.16-5.17 & Figure 5.10)**. One line was impenetrant (OX5241F3); three were fully penetrant but not repressible (OX5241AB1, AC2 and AK3). Full penetrance of OX5241AB1 was not expected, because its transgenic male founder was semi-fertile. The reason for this discrepancy was not obvious, because all previous male sterility phenotypes were generationally stable. Ccprot2-short 5'UTR-FokI (OX5241) appeared stronger than Ccprot1-full 5'UTR-FokI (OX5195). All lines of OX5241 demonstrating a reduction in egg viability were > 99% penetrant (only 1/5 lines for OX5195); and semi-penetrant lines were not observed (2/5 lines for OX5195). No further assessment was performed because the lines were not commercially valuable. We concluded that Ccprot2-short 5'UTR-FokI requires modification if applied further; and that uncharacterised factors underpinning a generational variation in transgenic phenotype are possible (OX5241AB1).

Table 5.16 Penetrance and repressibility of male sterility in OX5241 heterozygous males

Line	**Mean hatch rate (± SE)**		**Penetrance**			**Repressibility**		
	NT	**T**	%	X^2	$P_{[df]}$	%	X^2	$P_{[df]}$
OX5241AC2	0	0	100	176.3	**<0.001[1]**	0	153.0	**<0.001[1]**
OX5241AB1	0.3 ± 0.33	0.3 ± 0.33	99.6	175.1	**<0.001[1]**	0.38	151.9	**<0.001[1]**
OX5241AK3	0.7 ± 0.47	0	99.3	173.5	**<0.001[1]**	0	153.0	**<0.001[1]**
OX5241F3	92.3 ± 1.54	89.3 ± 1.78	1.4	0.150	$0.698_{[1]}$	100	0.320	$0.572_{[1]}$
WT	93.7 ± 1.41	86.7 ± 1.96						

Penetrance and repressibility were calculated relative to their respective control hatch rate, WT [NT] or [T]. Significance values were from chi-square tests of the numbers of hatched and unhatched eggs, relative to the respective values for the control (WT [NT] or [T]).

Table 5.17 Extent of repressibility of male sterility in OX5241 lines

Line	**Mean difference of hatch rates [NT – T] (%)**	**Significance**	
		X^2	$P_{[df]}$
OX5241AC2	0	0	$1_{[1]}$
OX5241AB1	0	0	$1_{[1]}$
OX5241AK3	-0.7	0.70	$0.40_{[1]}$
OX5241F3	-3.0	0.53	$0.46_{[1]}$
WT	-7.0	2.77	$0.10_{[1]}$

The difference of the mean hatch rate for [NT] and [T] reared males of each line was calculated. Significance testing was performed with chi-square tests of the numbers of hatched and unhatched eggs, between the [NT] and [T] reared individuals of each line. Penetrant lines were not repressible.

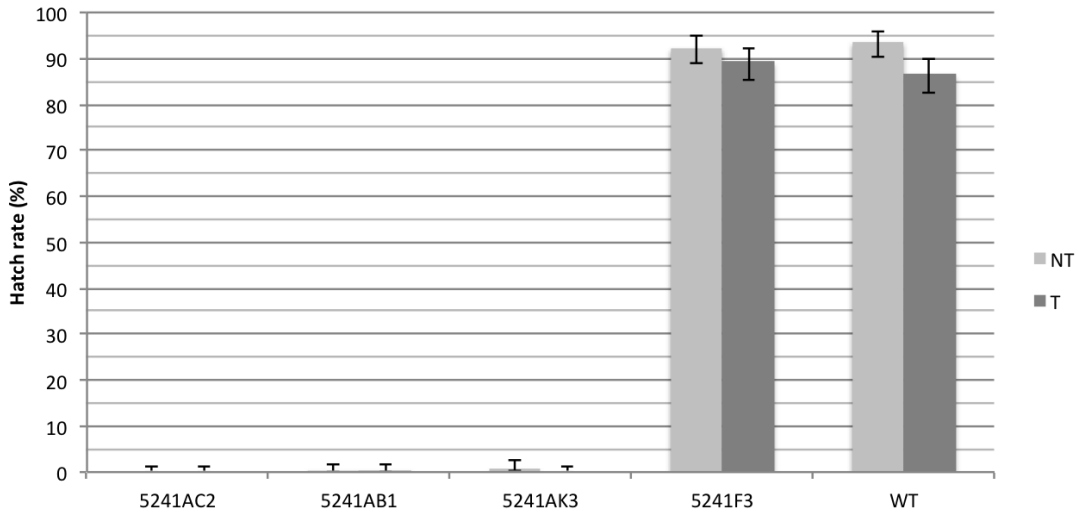


Figure 5.10. OX5241 (Ccprot2-short 5' UTR-FokI) heterozygous males demonstrate an irrepressibly penetrant male sterile phenotype. Egg hatch assay on progeny of OX5241 transgenic male individuals. OX5241F3 was impenetrant. OX5241AB1, AC2 & AK3 were highly penetrant but not repressible. Error bars: 95% CI.

5.4 Heterozygous OX5242 (Ccprot1-short 5'UTR-FokI) males have a mostly repressible sterility phenotype

5.4.1 Establishment of OX5242 transgenic lines

Microinjection (**Table 5.18**), backcrossing to WT (**Table 5.19**), screening for transient expression of the transformation marker, and Mendelian assessment of transgenic lines were essentially as described for OX5195/5241. Buffered mixes contained tetracycline (100 ng/µl), OX5242 (600 ng/µl), and *piggyBac* DNA helper OX3022 (300 ng/µl). RNA helper OX3081 was not used (there was no high quality RNA available at the time). Transient AlMAct-dsRed2 expression was observed in G_0 pupae, indicating successful microinjection. Adult survival (25%) was exactly average, compared to typical results for injection of Medfly (Gregory et al., 2016). Two rounds of injection were performed, denoted (1) or (2). Transformation efficiency was very poor in the first injection set (0.23%). There was insufficient time to assess OX5242(2) lines.

Table 5.18 Microinjection logistics for OX5242

Round	Embryos	Larvae	Pupae	Adults	Lines
1	1100	648 (59%)	463 (42%)	422 (38%)	1 (0.23%)
2	2210	887 (40%)	482 (22%)	404 (18%)	3 (0.74%)
Both	**3310**	**1535 (46%)**	**945 (29%)**	**826 (25%)**	**4 (0.48%)**

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (transgenic lines/G_0 adults crossed).

Table 5.19 G0 backcrosses to establish OX5242 lines

OX5242(1)A	8 ♂ x 30 WT ♀	OX5242(1)B	7 ♂ x 30 WT ♀	OX5242(1)C	20 ♀ x 10 WT ♂
OX5242(1)D	21 ♀ x 10 WT ♂	OX5242(1)E	21 ♀ x 10 WT ♂	OX5242(1)F	21 ♀ x 10 WT ♂
OX5242(1)G	20 ♀ x 10 WT ♂	**OX5242(1)H**	**20 ♀ x 10 WT ♂**	OX5242(1)I	20 ♀ x 10 WT ♂
OX5242(1)J	11 ♂ x 30 WT ♀	OX5242(1)K	11 ♂ x 30 WT ♀	OX5242(1)L	11 ♂ x 30 WT ♀
OX5242(1)M	11 ♂ x 30 WT ♀	OX5242(1)N	11 ♂ x 30 WT ♀	OX5242(1)O	11 ♂ x 30 WT ♀
OX5242(1)P	10 ♂ x 30 WT ♀	OX5242(1)Q	10 ♂ x 30 WT ♀	OX5242(1)R	10 ♂ x 30 WT ♀
OX5242(1)S	10 ♂ x 30 WT ♀	OX5242(1)T	10 ♂ x 30 WT ♀	OX5242(1)U	10 ♂ x 30 WT ♀
OX5242(1)V	18 ♀ x 10 WT ♂	OX5242(1)W	18 ♀ x 10 WT ♂	OX5242(1)X	18 ♀ x 10 WT ♂
OX5242(1)Y	9 ♂ x 30 WT ♀	OX5242(1)Z	9 ♂ x 30 WT ♀	OX5242(1)AA	9 ♂ x 30 WT ♀
OX5242(1)AB	9 ♂ x 30 WT ♀	OX5242(1)AC	10 ♂ x 30 WT ♀	OX5242(1)AD	20 ♀ x 10 WT ♂
OX5242(1)AE	8 ♂ x 30 WT ♀	OX5242(1)AF	10 ♂ x 30 WT ♀	OX5242(2)A	10 ♂ x 30 WT ♀
OX5242(2)B	10 ♂ x 30 WT ♀	OX5242(2)C	8 ♂ x 30 WT ♀	OX5242(2)D	16 ♀ x 8 WT ♂
OX5242(2)E	16 ♀ x 8 WT ♂	OX5242(2)F	10 ♂ x 30 WT ♀	**OX5242(2)G**	**10 ♂ x 30 WT ♀**
OX5242(2)H	**10 ♂ x 30 WT ♀**	OX5242(2)I	10 ♂ x 30 WT ♀	OX5242(2)J	10 ♂ x 30 WT ♀

OX5242(2)K	10 ♂ x 30 WT ♀	OX5242(2)L	8 ♂ x 30 WT ♀	OX5242(2)M	20 ♀ x 10 WT ♂
OX5242(2)N	18 ♀ x 10 WT ♂	OX5242(2)O	18 ♀ x 10 WT ♂	**OX5242(2)P**	**18 ♀ x 10 WT ♂**
OX5242(2)Q	9 ♂ x 30 WT ♀	OX5242(2)R	8 ♂ x 30 WT ♀	OX5242(2)S	8 ♂ x 30 WT ♀
OX5242(2)T	8 ♂ x 30 WT ♀	OX5242(2)U	12 ♀ x 10 WT ♂	OX5242(2)V	11 ♀ x 10 WT ♂
OX5242(2)W	30 ♀ x 10 WT ♂	OX5242(2)X	10 ♂ x 30 WT ♀	OX5242(2)Y	10 ♂ x 30 WT ♀
OX5242(2)Z	9 ♀ x 5 WT ♂	OX5242(2)AA	7 ♂ x 21 WT ♀	OX5242(2)AB	10 ♂ x 30 WT ♀
OX5242(2)AC	10 ♂ x 30 WT ♀	OX5242(2)AD	23 ♀ x 12 WT ♂	OX5242(2)AE	2 ♂ x 15 WT ♀
OX5242(2)AF	10 ♂ x 24 WT ♀	OX5242(2)AG	9 ♀ x 5 WT ♂	OX5242(2)AH	8 ♂ x 25 WT ♀
OX5242(2)AI	7 ♂ x 25 WT ♀	OX5242(2)AJ	11 ♀ x 10 WT ♂		

Underlined crosses gave rise to transgenic lines.

5.4.2 Heterozygous OX5242(1)H1 males demonstrate penetrant and mostly repressible sterility

The single transgenic G_1 individual isolated from the first round of microinjections (OX5242(1)H1) was a fertile female. The second round of injection was not assessed at the time of writing. Mendelian analysis was performed using a transgenic G_2 male backcrossed to WT (**Table 5.20**). G_2 females were backcrossed to WT males and filtered on- and off-tetracycline to assess G_3 progeny for a penetrant and repressible male sterility phenotype by egg hatch assay, as described for OX5241. 24 hour egg collections (100 eggs) were performed on day 5, 6 and 7 post-cross; significance testing was as previously described. The OX5242(1)H1 male sterility phenotype was penetrant (99%) and mostly repressible (81%); the reductions in egg hatch rate of progeny of transgenic males reared off-tet and on-tet were significant ($p < 0.001$) relative to off-tet and on-tet reared WT controls (**Table 5.21**, **Table 5.22**, **Figure 5.11**). Mean hatch rates differed ($p < 0.001$) between off-tet and on-tet reared OX5242(1)H1, but not WT, indicating that repressibility was significant but incomplete, and that viability differences were not mediated by an independent effect of tetracycline itself.

Table 5.20 Mendelian analysis of OX5242(1)H1

Line	Sex	Copy number		Sex-linkage		Summary	
		Transgenic (%)	n	Ratio (M/F)	n	Copies	Location
OX5242(1)H1	♂	47	675	1.0	281	1	Autosome

Table 5.21 Penetrance and repressibility of male sterility in OX5242(1)H heterozygous males

Line	Mean hatch rate (± SE)		Penetrance			Repressibility		
	NT	T	%	X^2	$P_{[df]}$	%	X^2	$P_{[df]}$
OX5242(1)H	1.3 ± 0.66	73.7 ± 2.54	99	169.8	**< 0.001**$_{[1]}$	81	10.3	**0.001**$_{[1]}$
WT	93.3 ± 1.44	91.0 ± 1.65						

Penetrance and repressibility were calculated relative to their respective control hatch rate, WT [NT] or [T]. Significance values were from chi-square tests of the numbers of hatched and unhatched eggs, relative to the respective values for the control (WT [NT] or [T]).

Table 5.22 Extent of repressibility of male sterility in OX5242 lines

Line	Mean difference of hatch rates [NT – T] (%)	Significance	
		X^2	$P_{[df]}$
5242(1)H	72.3	111.8	**< 0.001**$_{[1]}$
WT	-2.3	0.37	0.55

The difference of the mean hatch rate for [NT] and [T] reared males of each line was calculated. Significance testing was performed with chi-square tests of the numbers of hatched and unhatched eggs, between the [NT] and [T] reared individuals of each line.

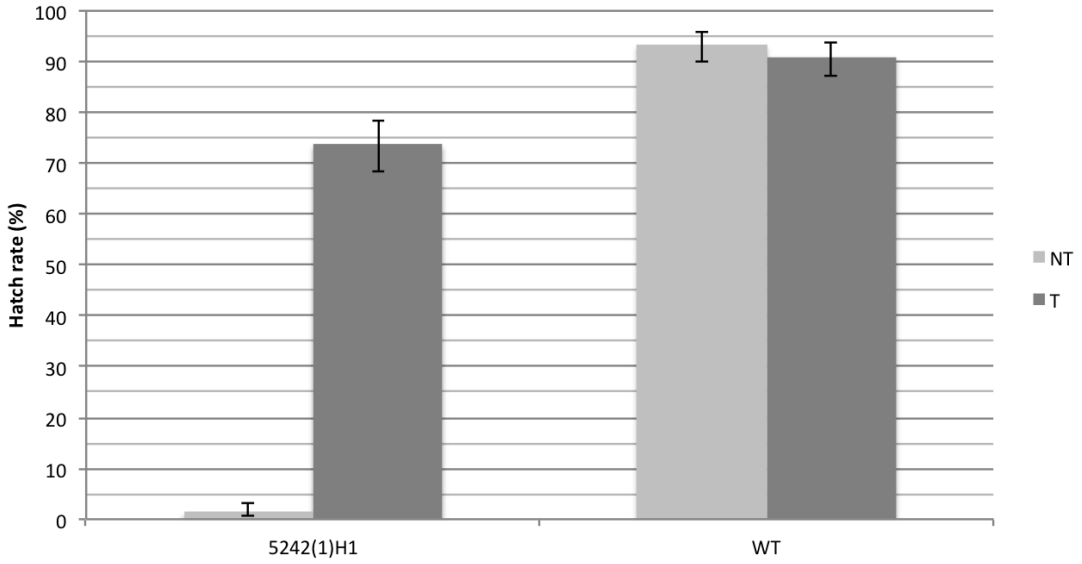


Figure 5.11. OX5242(1)H1 (Ccprot1-short 5'UTR-FokI) heterozygous males demonstrate a penetrant male sterility phenotype with nearly complete repression (81%). Egg hatch assay on progeny of OX5242(1)H1 transgenic male individuals. Error bars: 95% CI.

5.4.3 Heterozygous OX5242(1)H1 males transfer fluorescently marked sperm to mated females, but there is a reduction in quantity and motility

The nuclear localisation of the Ccprot1-zsGreen fluorescent sperm marker, and the quantity and motility of OX5242(1)H1 sperm transferred upon mating, were next assessed. Equivalent crosses to the egg hatch assay were initiated. Spermathecae of the WT females were dissected 7 days later at Cardiff University, as previously described for OX5195 (**Section 5.2.3**). We estimated the quantity of sperm present and their motility and appearance (ten individuals from each group). Sperm transfer and motility were markedly reduced in OX5242(1)H1[NT] relative to OX5242(1)H1[T], WT[NT] and WT[T] (**Figure 5.12)**. However, the transfer of detectably marked sperm was an improvement on the prior design (OX4718). For the OX5242(1)H1[NT] crosses, sperm were detected in 14/14 instances (> 100 sperm in 6/14 instances); for OX4718A-res [NT], sperm were detected in 4/16 instances (> 100 sperm were not detected in any instance). For reference, in the WT[NT] and WT[T] crosses, >100 sperm were detected in 9/10 and 10/10 instances, respectively. We did not detect any effects on the morphology of sperm of OX5242(1)H1 males. In some instances, sperm that appeared to have been crushed by the cover slip were observed; but these were also present in the WT samples. It would be interesting to perform further investigation at a higher magnification, and with a haemocytometer or similar device that would not risk crushing any cells under investigation, to completely exclude the possibility of morphological differences.

For the OX5242(1)H1[T] crosses, sperm were observed in every instance (12/12); more than 100 were present in 11/12 instances. This provided further evidence that reducing the expression of Ccprot1-short 5'UTR-FokI increased the quantity of sperm transferred upon mating. Consistent with the incomplete repression observed in the egg hatch assay, the number of sperm transferred appeared to be slightly reduced relative to the WT[T] crosses. However, it should be noted that it was not possible to precisely confirm a non-profound effect on the quantity of sperm transfer under these experimental conditions (squashes of spermathecae containing live sperm, directly observed at 10-40x magnification). More precise quantification is possible with stained samples under high magnification (Taylor et al., 2000). However, the technique is laborious and prone to inaccuracy due to the tendency of sperm to aggregate, and the necessity to accurately dilute samples for counting (Yuval et al., 1996). PCR-based strategies to quantify sperm by amplification of male-specific sequences are known, for

instance in the Tephritid pest *A. suspensa* (Fritz et al., 2010), (Doyle et al., 2011). Should it be necessary to correlate the number of sperm transferred with female tendency to re-mate, it would be interesting to develop a similar method for Medfly, which does not appear to exist currently.

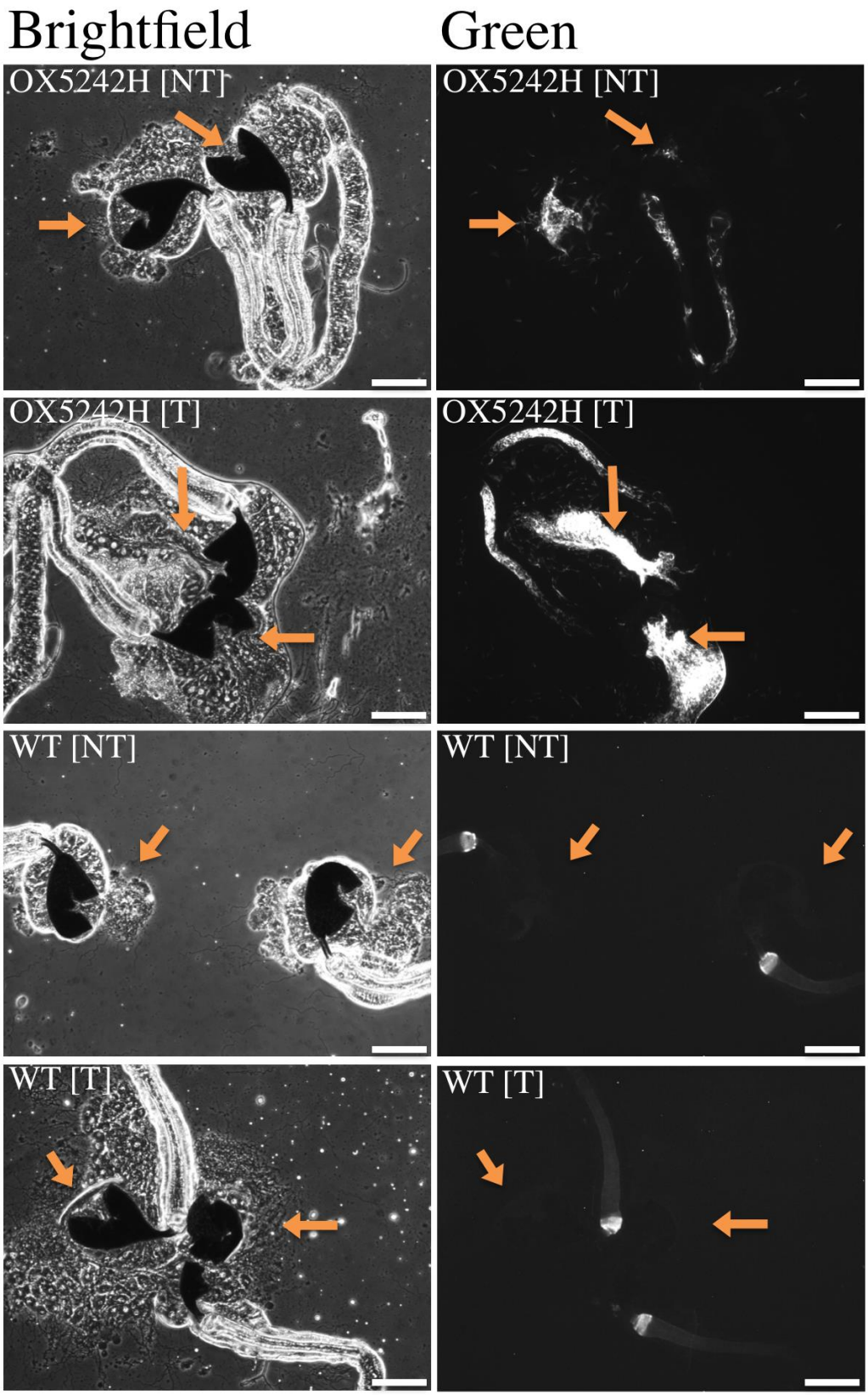


Figure 5.12. Sperm transfer in OX5242(1)H heterozygotes is negatively affected by expression of Ccprot1-short 5'UTR-FokI. Fluorescence and phase contrast microscopy of dissected spermathecae from WT females mated to WT or OX5242(1)H males, comparing expression in the induced (off-tet [NT]) and repressed (on-tet [T]) states. Females mated to OX5242(1)H [NT] demonstrate fewer visible sperm (block arrows) relative to females mated to OX5242(1)H [T], WT [NT] or WT[T]. Moving sperm were rarely detected in females mated to OX5242(1)H [NT]; all other groups appeared normal. Scale bars: 100 μm.

5.4.4 The OX5242(1)H1 transgenic insertion is homozygous viable

Tet-reared OX5242(1)H1 heterozygous males (n=3) and females (n=3) were crossed and F_1 progeny filtered from two egg collections, on-tet. Chi-square testing was performed on the observed and expected ratios of transgenic progeny (25% WT progeny are expected from a cross of two individuals heterozygous for a dominant trait). Both rears were assessed for significant variance prior to pooling; none was detected ($X^2 = 1.553$, df = 1, p = 0.2127). The results indicated homozygous viability; about 75% of progeny were transgenic (**Table 5.23**).

Table 5.23 Homozygous viability of OX5242(1)H1

	Fluorescent marker inheritance in F_1 progeny						
	Fluorescent		**Not fluorescent**		**Total**	**X^2**	**$P_{[df]}$**
	Observed	Expected	Observed	Expected			
OX5242(1)H	201 (78.5%)	192 (75%)	55 (21.5%)	64 (25%)	256	0.887	$0.35_{[1]}$

5.5 Heterozygous OX5257V1 (Dmprot2-chimeric-new-FokI) males have a mostly repressible male sterility phenotype

OX5257 (Dmprot2-chimeric-new-FokI) was generated as a backup to the Ccprot1-short 5'UTR-FokI (OX5242) and Ccprot2-short 5'UTR-FokI (OX5241) constructs, to mediate the risk of sequence elements behaving in unpredicted ways. This was a modification of a previously evaluated system, Dmprot2-chimeric-old-mCherry-FokI (OX4718, **Figure 3.4**), which featured penetrant and repressible sterility but lacked motile, fluorescently marked sperm in most instances. Fluorescent sperm marking was engineered by addition of the Ccprot1-ZsGreen system, and removal of mCherry from the Dmprot2-chimeric-old-**mCherry**-FokI component of OX4718, which functioned poorly in prior investigation (**Figure 3.5**). This was expected to provide fluorescent sperm marking and maintain the prior penetrant and repressible male sterility phenotype. To avoid abolishing this phenotype, only two minimal changes were performed, as previously described: (1) SV40 3'UTR was substituted for Ccprot2 3'UTR and (2) the non-functional mCherry marker was removed (**Figure 5.13**).

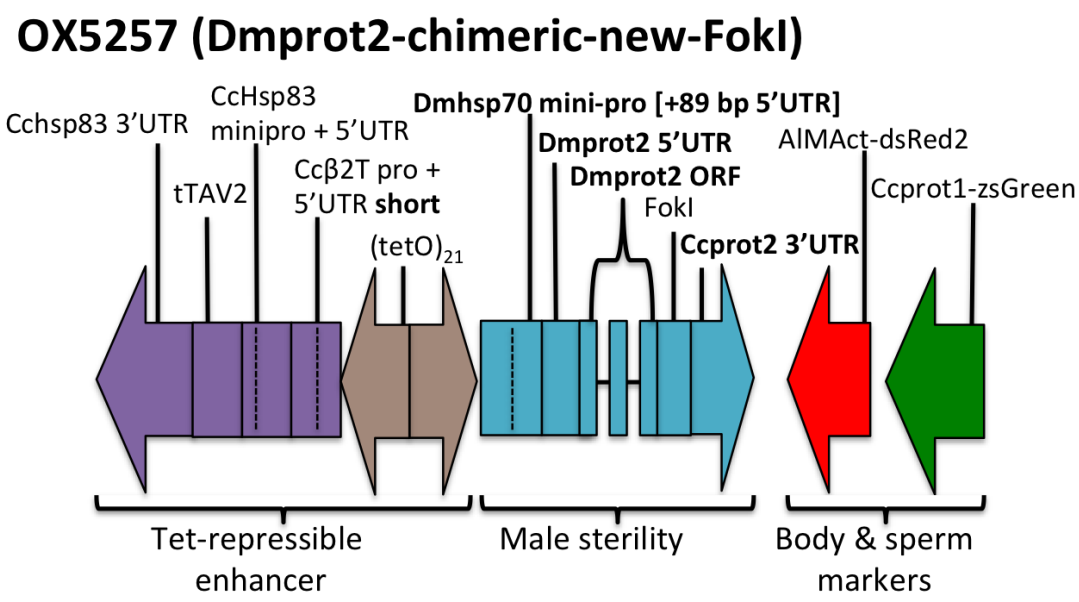


Figure 5.13. OX5257 expression construct diagram. Note that this incorporates a longer Dmhsp70 5'UTR (89 bp) relative to the other three constructs (OX5195, OX5241 & OX5257). The body marker (AlMAct promoter-AlMAct 5' UTR-dsRed2-AlMAct 3' UTR) and sperm marker (Ccprot1 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1 3'UTR) were as previously described. Dashes indicate promoter-5'UTR boundary. Note: piggyBac ends omitted from this four-ended vector (full diagram shown in Figure 2.2).

5.5.1 Establishment of OX5257 transgenic lines

Microinjection (**Table 5.24**), backcrossing to WT (**Table 5.25**), screening for transient expression of the transformation marker, and Mendelian assessment of transgenic lines were essentially as described for OX5195/5241/5242. Buffered mixes contained tetracycline (100 ng/µl), OX5242 (600 ng/µl), and *piggyBac* DNA helper OX3022 (300 ng/µl). As for OX5241, RNA helper OX3081 was not used. Transient AlMAct-dsRed2 expression was observed in G_0 pupae. Adult survival (10%) was substantially lower than average, compared to typical results for injection of Medfly (25%) (Gregory et al., 2016). Phenotypic analysis was largely as for OX5242; investigation of three lines (OX5257G, V, & AX) was initiated at the time of writing.

Table 5.24 Microinjection logistics for OX5257

Embryos	Larvae	Pupae	Adults	Lines
6315	1334 (21%)	693 (11%)	607 (10%)	4 **(0.7%)**

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (transgenic lines/G_0 adults crossed).

Table 5.25 G0 backcrosses to establish OX5257 lines

OX5257A	7 ♂ x 21 WT ♀	**OX5257B**	**6 ♀ x 6 WT ♂**	OX5257C	5 ♂ x 15 WT ♀
OX5257D	6 ♀ x 6 WT ♂	OX5257E	2 ♂ x 6 WT ♀	OX5257F	4 ♀ x 4 WT ♂
OX5257G	**11 ♂ x 30 WT ♀**	OX5257H	12 ♀ x 6 WT ♂	OX5257I	6 ♂ x 30 WT ♀
OX5257J	7 ♂ x 30 WT ♀	OX5257K	6 ♀ x 3 WT ♂	OX5257L	7 ♂ x 30 WT ♀
OX5257M	10 ♀ x 5 WT ♂	OX5257N	11 ♂ x 30 WT ♀	OX5257O	10 ♀ x 5 WT ♂
OX5257P	4 ♂ x 15 WT ♀	OX5257Q	7 ♀ x 7 WT ♂	OX5257R	9 ♂ x 30 WT ♀
OX5257S	8 ♂ x 30 WT ♀	OX5257T	10 ♀ x 5 WT ♂	OX5257U	7 ♂ x 30 WT ♀
OX5257V	**7 ♀ x 4 WT ♂**	OX5257W	6 ♂ x 18 WT ♀	OX5257X	11 ♀ x 6 WT ♂
OX5257Y	12 ♂ x 30 WT ♀	OX5257Z	17 ♀ x 9 WT ♂	OX5257AA	11 ♂ x 30 WT ♀
OX5257AB	10 ♂ x 30 WT ♀	OX5257AC	13 ♀ x 7 WT ♂	OX5257AD	8 ♂ x 30 WT ♀
OX5257AE	8 ♀ x 4 WT ♂	OX5257AF	12 ♂ x 30 WT ♀	OX5257AG	6 ♀ x 3 WT ♂
OX5257AH	8 ♂ x 30 WT ♀	OX5257AI	9 ♂ x 30 WT ♀	OX5257AJ	16 ♀ x 6 WT ♂
OX5257AK	8 ♂ x 25 WT ♀	OX5257AL	7 ♀ x 4 WT ♂	OX5257AM	8 ♂ x 25 WT ♀
OX5257AN	7 ♂ x 25 WT ♀	OX5257AO	18 ♀ x 9 WT ♂	OX5257AP	10 ♂ x 30 WT ♀
OX5257AQ	10 ♂ x 30 WT ♀	OX5257AR	18 ♀ x 9 WT ♂	OX5257AS	10 ♂ x 30 WT ♀
OX5257AT	10 ♂ x 30 WT ♀	OX5257AU	10 ♂ x 30 WT ♀	OX5257AV	11 ♂ x 30 WT ♀
OX5257AW	14 ♀ x 10 WT ♂	**OX5257AX**	**17 ♀ x 10 WT ♂**	OX5257AY	5 ♂ x 15 WT ♀
OX5257AZ	8 ♂ x 30 WT ♀	OX5257BA	7 ♂ x 30 WT ♀	OX5257BB	11 ♀ x 10 WT ♂
OX5257BC	14 ♀ x 7 WT ♂	OX5257BD	10 ♂ x 29 WT ♀	OX5257BE	4 ♂ x 12 WT ♀
OX5257BF	8 ♂ x 30 WT ♀	OX5257BG	7 ♂ x 30 WT ♀	OX5257BH	7 ♂ x 30 WT ♀
OX5257BI	12 ♀ x 7 WT ♂	OX5257BJ	12 ♀ x 7 WT ♂	OX5257BK	20 ♀ x 10 WT ♂
OX5257BL	10 ♂ x 30 WT ♀	OX5257BM	5 ♂ x 15 WT ♀		

Underlined crosses gave rise to transgenic lines.

5.5.2 OX5257 transgenic G_1 male individuals are fertile

G_1 transgenic backcrosses were monitored for fertility and the presence of fluorescently marked sperm (**Table 5.26, Figure 5.14)**. All microscopy was performed at Oxitec, as previously described. Males and females were fertile in all instances. Stable heterozygous transgenic lines were established from individuals OX5257B1, G1, V1 and AX1. Mendelian properties of each insertion were assessed **(Table 5.27**). There was insufficient time to investigate in detail the post-mating sperm transfer phenotype at Cardiff University. The OX5257G1 insertion was y-linked and not assessed further; evaluation proceeded for OX5257B1, V1 & AX1.

Table 5.26 Fertility of OX5257 transgenic G_1 individuals

Individual	Sex	Fertile	Sperm marking	Apparent phenotype
OX5257G1	♂	Yes	No	Impenetrant male sterility with no fluorescent sperm marking
OX5257G2	♂			
OX5257V1	♂		Yes	Penetrant and repressible male sterility with fluorescent sperm marking
OX5257AX1	♂			
OX5257B1	♀			
OX5257B2	♀			
OX5257V2	♀			
OX5257AX2	♀			

Permanent lines were established from boldface entries.

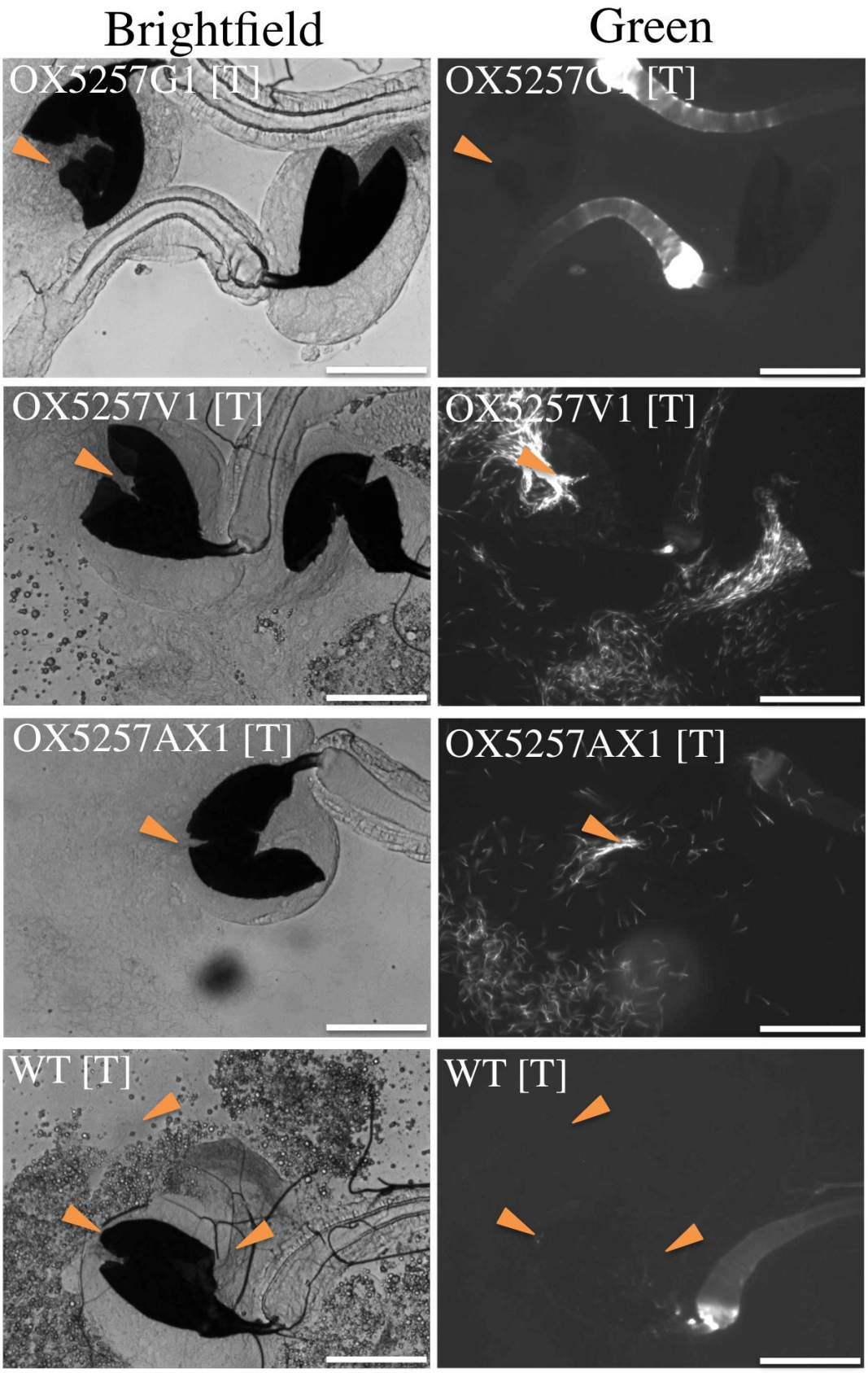


Figure 5.14. OX5257 (Dmprot2-chimeric-new-FokI) G_1 transgenic individuals transfer fluorescently marked sperm (Ccprot1-zsGreen) to females after mating. OX5257 G_1 trangenic males (tet-reared) were mated to WT females; spermatheae were dissected. All individuals were fertile and transferred sperm to females (**shaded arrowheads**); marking was observed in OX5257V1 and AX1. Penetrance and repressibility were investigated in the next generation. Scale bars: 100 μm.

Table 5.27 Mendelian analysis of OX5257 transgenic lines

Line	Sex	Copy number		Sex-linkage		Summary	
		Transgenic (%)	n	Ratio (M/F)	n	Copies	Location
OX5257B1	♂						
OX5257G1	♂			Male-only	49		Y
OX5257V1	♂	46	37	0.86	13	1	Autosome
OX5257AX1	♂						

OX5257G1 was not assessed for copy number because it was Y-linked. Time was insufficient to assess OX5257B1 and AX1.

5.5.3 OX5257V1 heterozygous males demonstrate penetrant and mostly repressible sterility

Assessment of the penetrance and repressibility of male sterility was performed by egg hatch assay, largely as described for OX5242(1)H1. F_0 crosses were 10 tet-reared OX5257 males to 20 non-tet reared WT females. All subsequent crosses, egg collections and statistical assessment were as described for OX5242(1)H1. There was only sufficient time to assess OX5257V1 (**Table 5.28, Figure 5.15**), which was fully penetrant and mostly repressible (71%). Mean hatch rates differed significantly between progeny of tet-reared and non-tet reared males of OX5257V1, but not WT, indicating that there was no significant independent effect of tetracycline on viability (**Table 5.29**). OX5257V1 demonstrated a similar profile to OX5242(1)H1; slightly more penetrant (1%) but less repressible (10%). Although OX5242(1)H1 [Ccprot1-short 5'UTR-FokI] and OX5257V1 [Dmprot2-chimeric-new-FokI] are both promising for commercial use, it remains possible that a superior, fully repressible line will be isolated from the strains not yet evaluated.

Table 5.28 Penetrance and repressibility of male sterility in OX5257 heterozygous males

Line	Mean hatch rate (± SE)		Penetrance			Repressibility		
	NT	T	%	X^2	$P_{[df]}$	%	X^2	$P_{[df]}$
OX5257V1	0	67.3 ± 2.71	100	188.4	**> 0.001**$_{[1]}$	71.1	24.2	**> 0.001**$_{[1]}$
WT	97 ± 0.98	94.6 ± 1.30						

Penetrance and repressibility were calculated relative to WT [NT] hatch rate. Significance values were from chi-square tests of the numbers of hatched and unhatched eggs, relative to the respective values for the control (WT [NT] or [T]).

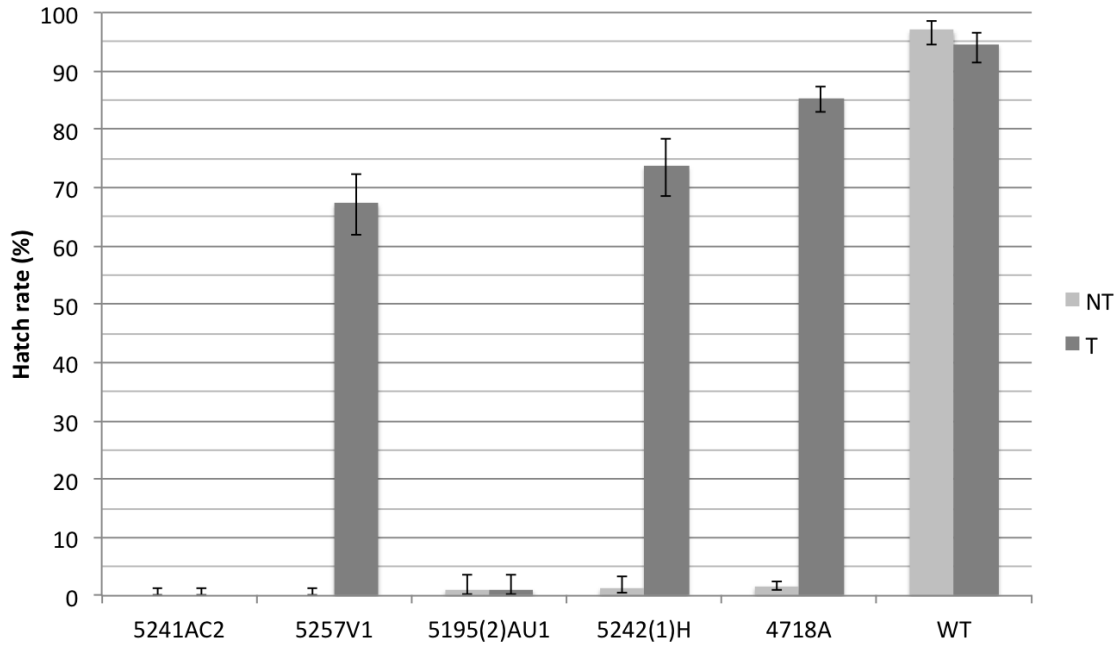


Figure 5.15. OX5257V1 heterozygous males demonstrate a penetrant male sterility phenotype with nearly complete repression (71%). Egg hatch assay on progeny of OX5257V1 transgenic male individuals demonstrates statistically significant reductions in hatch rate relative to control, for both the NT and T reared groups (partial repression of penetrant male sterility). Other lines of interest from this study are presented in order of penetrance: OX5241AC2 (Ccprot2-short-FokI); **OX5257V1** (Dmprot2-chimeric-new-FokI); OX5195(2)AU1 (Ccprot1-full-FokI); OX5242(1)H (Ccprot1-short-FokI); OX4718A (Dmprot2-chimeric-old-FokI). Error bars: 95% CI.

Table 5.29 Extent of repressibility of male sterility in OX5257 lines

Line	**Mean difference of hatch rates [NT – T] (%)**	**Significance**	
		X^2	$P_{[df]}$
5257V1	67.3	101.4	**> 0.001**$_{[1]}$
WT	-2.4	0.72	0.40$_{[1]}$

The difference of the mean hatch rates for [NT] and [T] reared males of each line was calculated. Significance values were from chi-square tests of the numbers of hatched and unhatched eggs, relative to the respective values for the control (WT [NT] or [T]).

5.6 Conclusions

This study attempted to generate a functional product candidate with repressible male sterility and fluorescent sperm marking, suitable for integration with the male-selecting strain OX3864A in a final, stacked trait product. A primary objective was to delay translation of the protamine-FokI effector protein by removal of the Dmhsp70 5'UTR element and inclusion of Medfly protamine 5'UTR sequences. This was expected to enhance the competitive phenotype of sterile sperm, which was severely affected in a prior transgenic strain (OX4718); few sperm were transferred to females after mating, and the majority did not swim. This was considered likely to reduce the suppressive ability of such a strategy in population control, because females would be

more likely to re-mate, potentially with wild males (Lin et al., 1996), (Mossinson and Yuval, 2003).

Four variant designs were tested, and two provided a line with the desired properties (**OX5242**: Ccprot1-short 5'UTR-FokI; **OX5257**: Dmprot2-chimeric-new-FokI; OX5195: Ccprot1-full 5'UTR-FokI; and OX5241: Ccprot2-short 5'UTR-FokI). At the time of writing, OX5242(1)H1 met several preliminary criteria for an acceptable product (single autosomal insertion; penetrant and repressible male sterility; transfer of fluorescently marked sperm to females; and homozygous viability). OX5257V1 met the first two criteria, but neither homozygous viability nor the ability of the strain to transfer fluorescently marked sperm to females (when reared off-tet) have yet been assessed. This is vital, as a highly similar expression construct (OX4718) was associated with a reduction in the quantity of sperm transferred.

We had previously confirmed that fusion of Dmhsp70 promoter to Ccprot1 retained the translational expression profile conferred by Ccprot1 5'UTR (Chapter 4: OX5184 [DmHsp70 promoter [+21 bp 5'UTR]-Ccprot1-zsGreen]). However, when we altered this system to mediate repressible male sterility, we found that it was not repressible (OX5195: Ccprot1-full 5'UTR-FokI). This was not entirely surprising, as the reporter construct (OX5184) did not incorporate the tetracycline-repressible regulatory element (ie. placement of the Ccprot1-zsGreen system in a head-to-head configuration to the male germline switch, to put the tetO21 site upstream of the Ccprot1-zsGreen transcription unit, and thus facilitate tetracycline repression). Therefore, it would not have been possible to detect such an effect; it was an oversight to not investigate this at the time. A theoretical expression construct that would have facilitated this analysis is provided for context (**Figure 5.16**). This would have provided the benefits of directly confirming tetracycline-mediated repressibility (or lack thereof) in the male germline, with a simpler and more rapid analysis. This construct would include the previously validated transformation (AlMAct-dsRed2) and sperm markers (Ccprot1-zsGreen); and a reporter system designed to test a novel protamine for future use as a regulator of repressible male sterility: tetO21-Dmhsp70-novel protamine-mCherry). The level, timing and repressibility of tetO-protamine localised effector expression would be confirmed by differential expression of mCherry in the male germline (strong off-tet and weak or absent on-tet). This would allow the inference of phenotype immediately and directly (by fluorescent microscopy rather than egg hatch

assay), without the confounding factor of non-repressible male sterility (leading to line loss).

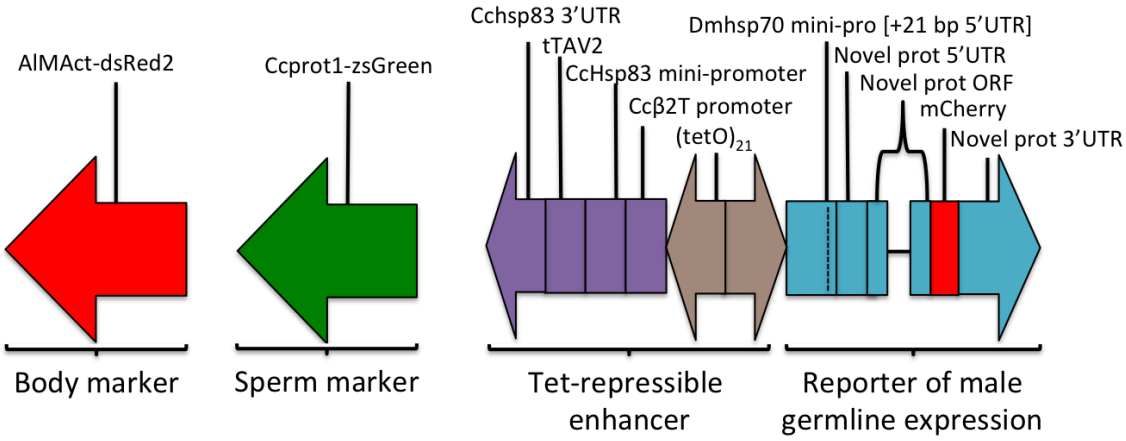


Figure 5.16. Reporter expression constructs to test tetracycline-repressible effector localisation (tetO21-Dmhsp70-protamine-mCherry-FokI) in the male germline. The construct would apply previously validated transformation (AlMAct-dsRed2) and sperm markers (Ccprot1-zsGreen); and a reporter system designed to test a novel protamine for future use as a regulator of repressible male sterility: tetO21-Dmhsp70-novel protamine-mCherry). The level, timing and repressibility of protamine-localised effector expression would be confirmed by differential expression of mCherry in the male germline (strong off-tet and weak on-tet). Dashes indicate promoter-5'UTR boundary. Note: piggyBac ends omitted from this hypothetical two-ended vector.

We concluded that the non-repressible male sterility phenotype of OX5195 (Ccprot1-full 5'UTR-FokI) was mediated by cryptic elements in the Ccprot1 5'UTR, which promoted transcription. This was based upon the observation that prior constructs applying a tetO21-Dmhsp70 minipromoter-Dmprot2-FokI fragment (eg. OX4353/4718) were fully repressible with equivalent tetracycline; that partial sensitivity to tetracycline was observed; that multiple transcriptional start sites were indicated by 5' RACE (**Section 5.2.7**); and that *D. melanogaster* genes are known with promoter elements in the 5'UTR (Kempe et al., 1993). This hypothesis was strongly supported by the subsequent isolation of repressible strains, after the Ccprot1 5'UTR was truncated (OX5242: Ccprot1-short 5'UTR-FokI). Therefore, this putative secondary promoter appeared to be trimmed to minimal activity, or brought into a spatially repressible conformation by its proximity to tetO21-Dmhsp70 minipromoter. Interestingly, shortening the Ccprot2 5'UTR fragment had no detectable effect; male sterility was completely penetrant and not repressed by tetracycline. It remains possible that further truncation could enhance repressibility whilst maintaining the favourable post-transcriptional control conferred by the Ccprot2 5'UTR. However, this was not pursued, as there is no commercial benefit to developing a redundant system at present.

We were surprised to find that a transgenic line with a strongly paternal effect lethal phenotype (OX5195(2)AU1: Ccprot1-full 5'UTR-FokI) did not generally arrest embryonic development at a pre-blastodermal stage. In *D. melanogaster*, a proportion of embryos derived from mutants for *maternal haploid* (22%) and *sésame* (72%) are able to proceed to very late stages of embryonic development (cuticle formation), despite essentially zero contribution of the paternal genome to development, and the frequent observation of aneuploidy (Loppin et al., 2000), (Loppin et al., 2001). Therefore, fragmentation of the paternal genome does not appear sufficient to block embryonic development in every instance. From the practical standpoint of using protamine-FokI expressing sterile males for Medfly population control, this is not a major issue. Only 1% of Ccprot1-FokI expressing embryos hatch into larvae (OX5195/5242), and at present, it appears that none survive to adulthood. Therefore, there would be minimal damage to fruit mediated by larval hatching, and no risk of transgenes propagating vertically in the wild. It is expected that the paternal effect of Ccprot1-short 5'UTR-FokI expression (OX5242), which has not yet been assessed, will be similar in the progeny of off-tet reared males. However, we expect minimal effects upon progeny of on-tet reared males (repression was successfully engineered). There was insufficient time to perform this analysis, but it will be particularly interesting to investigate the effects on very late development (48-72 hours), and to confirm that the rate of adult eclosion is 0%, as expected from preliminary data.

The effect of protamine-FokI expression on the quantity of sperm transferred after mating, and their motility, appears to be systematically negative; penetrance was associated with reduced quantity and motility of transferred sperm in three designs (OX4718: Dmprot2-chimeric-old-mCherry-FokI; OX5195: Ccprot1-full 5'UTR-FokI and OX5242: Ccprot1-short 5'UTR-FokI). This was not assessed in detail for OX5241 (Ccprot2-short 5'UTR-FokI) or OX5257 (Dmprot2-chimeric-new-FokI) at the time of writing. It therefore appears that considerable modification to the repressible male sterility expression system would be required to provide a sterile phenotype without any effect on the motility of sperm, or the number transferred after mating. An inverse association was observed between penetrance of male sterility and the quantity of sperm transferred, in an assessment of several OX5195 (Ccprot1-full 5'UTR-FokI) lines. This indicated that delayed translation was not sufficient to fully restore the fitness of protamine-FokI expressing sperm, which was unexpected. However, it is not entirely surprising, as Dmhsp70 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1

3'UTR (OX5184) was found to localise to spermatids which had not yet fully elongated (**Figure 4.14**). Even at this stage, extensive cleavage of nuclear DNA could be anticipated to disrupt nuclear shaping and subsequent individualisation. Sterilisation by irradiation is known to reduce the number of sperm in Medfly, and reduce the size of the sperm head (Seo et al., 1990), (McInnis, 1993). Notably, both irradiation and expression of protamine-FokI are known to induce double-strand breaks, though in the case of radiation, single-strand breaks and nucleotide damage also result (Fedrigo et al., 2012). We observed a clear reduction in the quantity of sperm, but no effect on the heads of sperm or their morphology were detected. It is possible that investigation at a higher magnification would be required to detect this, however.

We were surprised that the Dmprot2-chimeric-new-FokI system (OX5257) demonstrated incomplete repression in the single line tested, OX5257V1. A similar expression construct mediated penetrant and fully repressible sterility in the single strain tested (OX4718A-resolved; **Figure 3.5**). Relative to OX4718, several changes were made. First, the spatial orientation of elements on the OX5257 construct were changed. For instance, a Ccprot1-zsGreen fluorescent sperm marking system was added. This would not be expected to have an effect, because the relative orientation of the tetracycline-repressible switch and male sterility effector were retained. Additionally, mCherry was removed from the protamine-FokI effector. This might be expected to affect penetrance by altering the physical interaction with target DNA, but an effect on repressibility would not be expected. Finally, the 3'UTR attached to Dmprot2-FokI was altered from SV40 to Ccprot2. Because the 3'UTR is typically dispensible for appropriate post-transcriptional germline regulation in *Drosophila* (White-Cooper, 2009), it was assumed that this would not have an effect. It is not certain if this is a line-specific effect, as only OX5257V1 was tested at time of writing. Although repressibility was not attenuated by insertion in certain systems (Ccprot1-full 5'UTR-FokI [OX5195]), line-specific non-repressibility has been described elsewhere in a female-specific flightless mosquito line (Labbé et al., 2012). Consequently, it is possible that a fully repressible line of OX5242 or OX5257 may be obtained. Although repression is incomplete in OX5242(1)H1 and OX5257V1, it is adequate to facilitate mass-rearing of the strain, and therefore these designs remain commercially viable.

Despite the improvements to the transgenic expression systems for repressible male sterility described herein, substantial work remains in characterizing the

commercial suitability of these strains for population control of Medfly. These include an investigation of compatibility with the genetic male selection component (OX3864), a full assessment of mating competitiveness in the double homozygous state, and a comparison of life history traits (mass, flight ability, longevity, lifetime fecundity, mating competitiveness, and stress tolerance to starvation and thirst) against wildtype, to assess the effects of transgene expression on viability. We anticipate that a product line successfully meeting these criteria will be an invaluable tool for area-wide population control of Medfly, and may provide useful components and engineering principles for making equivalent strains in other pest insects.

Chapter 6 – Gene editing in Medfly with CRISPR

6.1 Introduction

6.1.1 Gene editing

The modification of a genome with target-specific effectors is a rapidly developing field with diverse applications in academic research (Wang et al., 2013), medicine (Gersbach and Perez-Pinera, 2014) and industry (van Erp et al., 2015). The effector is generally a DNA endonuclease that generates double-strand breaks. Subsequently, the target is modified by non-homologous end-joining (NHEJ) or homologous recombination (HR), in a cell-cycle dependent fashion (Saleh-Gohari, 2004), (Gasiunas and Siksnys, 2013), (Lin et al., 2014a). NHEJ is error-prone and frequently leaves an insertion-deletion (indel) footprint, useful for mutagenesis or targeted deletion. Homologous recombination repairs the DNA damage by copying a template of similar sequence. This allows precise genomic engineering of the repair outcome, facilitating diverse modifications ranging from a single nucleotide substitution to site-specific integration of a transgenic expression construct (Gratz et al., 2013). Emerging technologies may apply diverse, non-nuclease effectors such as transcriptional or epigenetic activators/repressors; this extends functional capability far beyond simple edits of genomic sequences (Larson et al., 2013), (Hilton et al., 2015). Three modular technologies have been widely applied (**Figure 6.1**): zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspersed palindromic repeats (CRISPR) (Gaj et al., 2013).

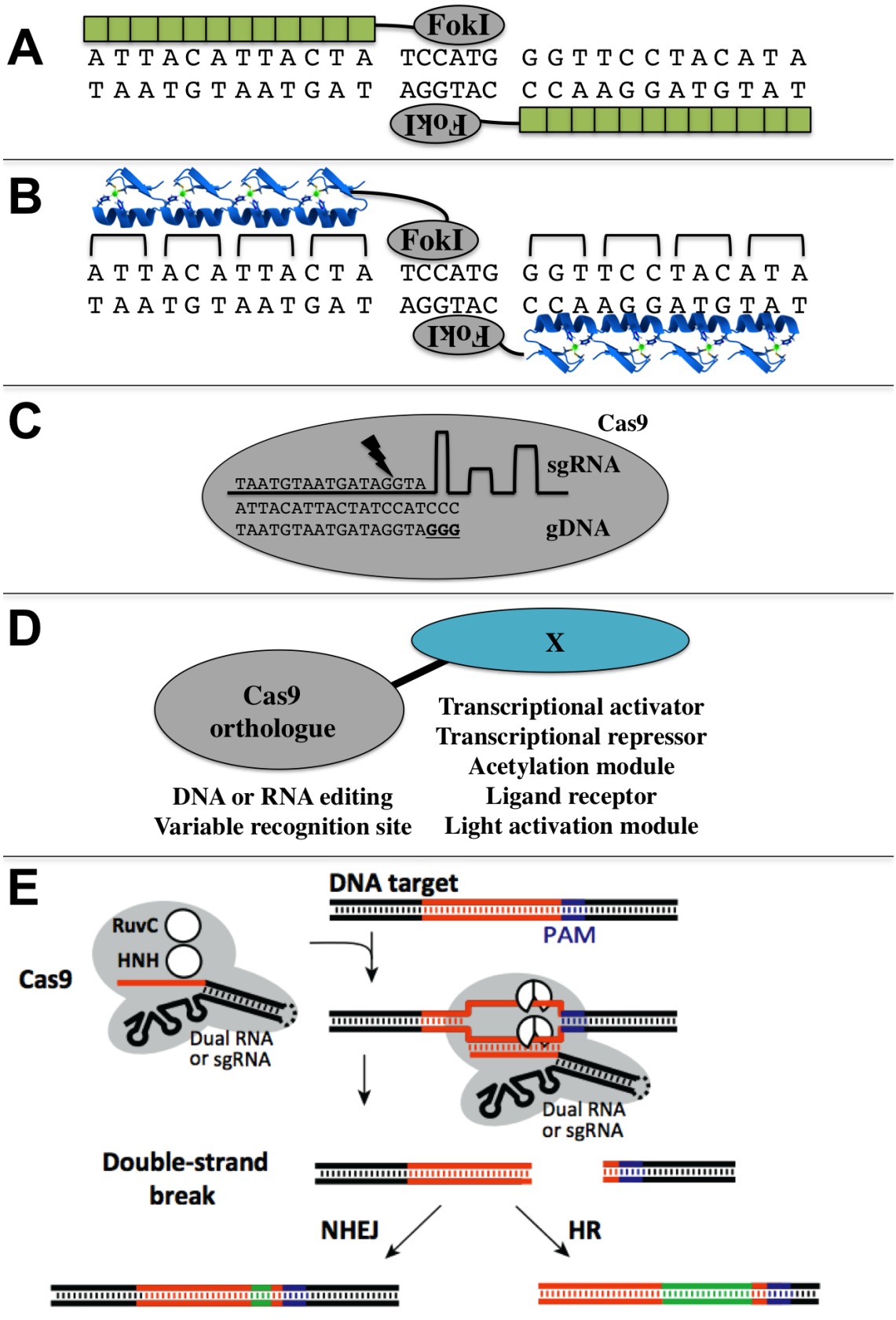


Figure 6.1. Overview of gene editing technologies. (**A-C**) Schematic diagram of target recognition by (**A**) TALENs, (**B**) ZFNs and (**C**) CRISPR. Lightning bolt indicates cleavage site. **(D)** Cas9 orthologues with specificity to either DNA or RNA, and variable target site recognition are known. Combinatorial fusion with effector modules facilitates development of diverse genetic study tools. (**E**) Utilisation of cellular repair mechanisms (non-homologous end joining [NHEJ] and homologous recombination [HR]), to facilitate sequence deletion or replacement. Image credit: D: Gasiunas & Siksnys (2013).

6.1.2 ZFNs and TALENs

ZFNs apply DNA binding modules from zinc-finger transcription factors: a 30 residue motif binds specifically to 3 bp of target sequence (Liu et al., 1997). The key programming benefit is the molecular separation of DNA-binding elements into discrete units, which are then placed in an array on the protein, separated by linkers. This facilitates development of targeted nucleases (Wah et al., 1997), (Gaj et al., 2013) or other cis-acting regulators. For each target site, a pair of ZFNs is designed that flanks the desired cut sequence. Dimerisation of the FokI cutting domains (one copy on each ZFN) generates an active endonuclease. Similarly, TALENs (transcription activator-like effector nucleases) are based on *Xanthomonas* DNA-binding proteins that modulate host gene expression with an N-terminal translocation domain, central DNA-binding domain and C-terminal transactivation domain (Morbitzer et al., 2010). The central region contains repeat variable dipeptides (RVDs), each specifying a single target nucleotide (Moscou and Bogdanove, 2009).

RVDs facilitate recognition of target nucleotides, with two exceptions. The last nucleotide is recognised by a half-repeat RVD and the first (usually thymine) by elements immediately upstream of the RVDs (Moscou and Bogdanove, 2009), (Miller et al., 2011). A different scaffold is required to target an alternative first nucleotide, because the mode of interaction is cryptic and not equivalent to the RVDs (Sanjana et al., 2012). Efficient designs have applied 9-19 RVDs per TALEN pair (Reyon et al., 2012). As with ZFNs, two adjacent sequences are typically targeted with a TALEN pair, directing the cut to the intervening sequence. However, single-chain TALEN designs with two intra-molecular FokI subunits have been described (Sun and Zhao, 2014). It is understood that target site, TALEN length and spacer distance can affect activity (Miller et al., 2011). Although a role of DNA-binding energy has been suggested (Guilinger et al., 2014a), a holistic model explaining the relative contribution of target sequence, chromatin structure, TALEN length and spacer distance remains to be developed.

6.1.3 CRISPR

CRISPR technology applies Cas9 nuclease and a guiding RNA complex. In native bacterial systems, CRISPR functions as an adaptive immune system (Brouns et al., 2008). CRISPR exists in most bacterial and archaeal species, demonstrating diverse modes of regulation (Makarova et al., 2013). Phylogenetic analysis suggests five major groups: classes II and V have been adapted for genetic engineering (Makarova et al.,

2015). Class II systems have been most widely applied, and are the focus of this study. In this system, cas-associated proteins cleave and genomically integrate invading DNA into operons with palindromic repeats, as protospacers (Jansen et al., 2002), (Makarova et al., 2011). Thereafter, transcription and processing leads to mature, target-specific CRISPR RNAs (crRNAs). A trans-activating CRISPR RNA (tracrRNA) molecule mediates three-way interaction with the mature crRNA and Cas9 nuclease, facilitating target binding and cleavage immediately upstream of the required protospacer adjacent motif (PAM) (Brouns et al., 2008), (Wiedenheft et al., 2012), (Tsai and Joung, 2016).

The targeting features of the tracrRNA and crRNA may be combined into a single guide RNA (sgRNA), simplifying genetic engineering (Jinek et al., 2012), (Bassett et al., 2013). This single molecule features invariant hairpins for interaction with Cas9, and a variable, target-specific motif. Targeting is mediated by complementary Watson-Crick binding of the sgRNA to genomic DNA. Simplicity of sgRNA synthesis (template-free PCR with a common reverse primer and target-specific forward primer, followed by *in vitro* transcription), facilitates powerful and cost-effective screens. In contrast, TALEN and ZFN technologies require unique vectors with difficult cloning, because repetitive codons specify single nucleotide readout (TALEN: 2 codons; ZFNs: 10 codons). Therefore, the simplicity, transferability, and expanding resource pool of CRISPR underpin its recognition as a major scientific innovation (Ledford, 2015).

The CRISPR system demonstrates unique practical constraints. Cutting can occur without perfect complementarity (mismatches to the 5' region of the sgRNA are often tolerated), contributing to off-target effects (Cradick et al., 2013), (Lin et al., 2014b). Recent improvements for enhanced specificity have been described, including a truncated sgRNA design (Fu et al., 2014), dual sgRNA targeting (Xu et al., 2014), and Cas9 proteins modified for reduced off-target activity (Slaymaker et al., 2016). Not all sequences can be targeted, because an NGG proto-spacer motif is usually required immediately downstream of the 20 nt seed sequence (Díez-Villaseñor et al., 2009). Activity is possible with nAG or nGA motifs, but is frequently reduced by 50% or more (Zhang et al., 2014). However, reprogramming without reduced activity is possible by applying orthologous Cas9 proteins with alternative proto-spacer recognition; for instance, the *C. jejuni* Cas9 PAM is nnnnACA (Fonfara et al., 2014). Further innovations include a modular toolkit of Cas9-effector fusions and Cas9 isoforms with

novel activity, notably an RNA-editing Cas9 (Doudna and Charpentier, 2014), (Abudayyeh et al., 2016). Consequently, the utility of the CRISPR platform is likely to increase as the field develops.

The simplest application of editing technologies is the modification of a sequence to study function, enabling sophisticated reverse genetics screens. Various recombination-mediated technologies for targeted editing have been developed in diverse organisms including bacteria (Yu et al., 2000), yeast (Matsuzaki et al., 1990), insects (Huang et al., 2009), and mice (Chen et al., 2001). However, these were frequently limited by low efficiency (Baena-Lopez et al., 2013) and poor transferability to other species, due to the application of species-specific elements such as endogenous recombinases. Nuclease-mediated double-stranded breaks enhance successful recombination (Saintigny, 2001), facilitating efficient sequence replacement or deletion in a variety of organisms. Alternative applications of gene-editing technologies have been described, typically applying nuclease-defective isoforms to localise effectors (**Table 6.1**).

Table 6.1: Selected applications of gene editing technologies

Application	Reference
RNA editing	(O'Connell et al., 2014)
High-throughput gene function screens	(Shalem et al., 2014)
Doxycycline inducible gene disruption	(Dow et al., 2015)
Organoid models of human diseases	(Freedman et al., 2015)
Gene drive for *Anopheles* control	(Hammond et al., 2015)
Targeted epigenetic modification	(Hilton et al., 2015)
Correction of muscular dystrophy phenotype in patient derived stem cells	(Li et al., 2015)
Germline correction of β-thalassemia	(Liang et al., 2015)
Light mediated gene induction	(Polstein and Gersbach, 2015)
Oncogenic enhancer screening	(Korkmaz et al., 2016)
Scalable protein localisation in single neurons	(Mikuni et al., 2016)
RNA tracking	(Nelles et al., 2016)

6.1.4 Objectives of the study

We intended to design a modular toolkit for precise genetic engineering with CRISPR, to optimise internal product development and confirm that the system would allow the targeted removal or modification of genes of interest. We were particularly interested in two related applications: transposon immobilisation by targeted removal of *piggyBac* transposition sequences and site-specific construct integration by homologous recombination. Other applications were possible, for instance the development of tetracycline-repressible targeted repressors of genes required for male fertility; this could be applied for population control systems similar to those described in the subsequent chapters. However, targeted repression of many of these genes (eg. *Cc-aly* or *Cc-topi*) would be expected to completely block the development of mature spermatozoa, which would be undesirable (Lin et al., 1996), (Mossinson and Yuval, 2003). However, this would not preclude the use of CRISPR technology to investigate novel sequences to regulate transgenic effectors in the male germline, or further develop the understanding of genetic regulators of male and female fertility in Medfly, for which essentially nothing has been experimentally validated.

A highly functional system for transposon immobilisation, applying nested *piggyBac* transposition, has been applied herein and elsewhere (Dafa'alla et al., 2006). However, practical difficulties are apparent, using the latest male-sterile candidate construct (**Chapter 5**: OX5257) as an example. First, four-ended *piggyBac* (4-PB) constructs are larger (4.6 kb associated with the two markers sequences at each end) and more repetitive (multiple *piggyBac* elements), than two-ended *piggyBac* (2-PB) equivalents. This increases the difficulty and cost of vector synthesis, and reduces transformation, because *piggyBac* integration efficiency is generally size-dependent (Handler and Ii, 1999), (Lorenzen et al., 2003). However, the relationship between size and transformation efficiency appears to be complex; the transformation efficiency of a large 2-PB construct (OX5173: 15 kb) tested in this chapter was acceptable (8%). In comparison, the 4-PB constructs (17-18 kb) evaluated previously (**Chapter 5**) were extremely difficult to transform, despite being only 20% larger (OX5195, OX5242, OX5257: 1% efficiency). This suggests that either the composite transposon is less efficiently transformed, or that the transformation efficiency rapidly decreases above 15 kb. The former is particularly interesting, because it was previously demonstrated that certain mini-transposons of the composite 4-PB vector can transposase more efficiently than others, even when larger (Condon et al., 2007). Therefore, it is possible that

reduced transformation efficiency in 4-PB constructs is mediated by the composite transposon structure, rather than size alone. Large injection cohorts (n > 2000), in one instance spanning a month (OX5257), yielded fewer than 3 lines.

A second issue is the presence of three fluorescent markers (OX5257: AlMAct-dsRed2; HR5IE1-AmCyan; OPIE2-ZsGreen) that are difficult to be independently distinguished in some instances. This complicates visual selection of individuals from which both flanking *piggyBac* elements have excised. In particular, the zsGreen marker was frequently misscored as absent, by multiple experimenters (false negatives were confirmed by PCR). Furthermore, the excision of transposable ends (resolution) requires three generations, and subsequent PCR validation. Finally, resolution has proved impossible for some lines, for unknown reasons. It is possible that the adjacent genomic sequence has an effect, explaining why some insertions are difficult or impossible to resolve. However, it is difficult to explain why integration could occur at a given site, but not subsequent re-transposition of the ends. It is therefore likely that the requirements for efficient initial integration, are different to the requirements for subsequent remobilisation of the *piggyBac* ends.

To address these issues, we propose three solutions (**Figure 6.2**). The first is largely independent of the methods applied in this chapter, but included for context: to remove the fluorescent marker sequences from the 4-PB vector, thereby substantially reducing its size. This has been successfully applied for *P. gossypiella* (pink bollworm) transgenic expression constructs at Oxitec, with one individual yielding 6% of progeny without *piggyBac* ends (Morrison, 2007). In Medfly, the rate of excision of one end or the entire transposon (both pairs of ends and the central element) was estimated at 7% for one line (Dafa'alla et al., 2006). However, the number of individuals retaining the central element (the desired outcome of excision), without both pairs of *piggyBac* ends, was not stated. Therefore, it is clear that PCR screening could be applied to select events where the insertion lacks *piggyBac* ends, but further experiments would need to be performed to determine the efficiency of the process.

Alternatively, a traditional two-ended *piggyBac* vector could be applied, with subsequent deletion of the transposable ends by CRISPR. It remains possible that validated CRISPR systems may not resolve certain insertions, due to local chromatin effects. Therefore, the relevance and efficiency of this proposed system would require

substantial characterisation. As a final alternative, site-specific integration could be applied; resolution would not be required, because it is transposon-free. There exist further theoretical advantages, assuming adequate efficiency. First, it would be possible to modify previously obtained lines (for instance, to add an additional transgenic effector, such as a fluorescent sperm marking system). Secondly, it would be possible to target constructs to validated expression sites. When developing transgenic, repressible male sterility systems, selecting phenotypically ideal lines from a panel of random insertions is the major time constraint. This is because most lines do not perform as well as required, due to effects of the insertion site (Schönig et al., 2011). Consequently, enhanced selection by targeted integration to a known site with strong expression of transgenic effectors in the male germline, would be advantageous. Several sites were characterised in this study (**Chapters 4-5**). Finally, there is no need to determine the insertion site; and a single insertion is guaranteed, a requirement of a final commercial product.

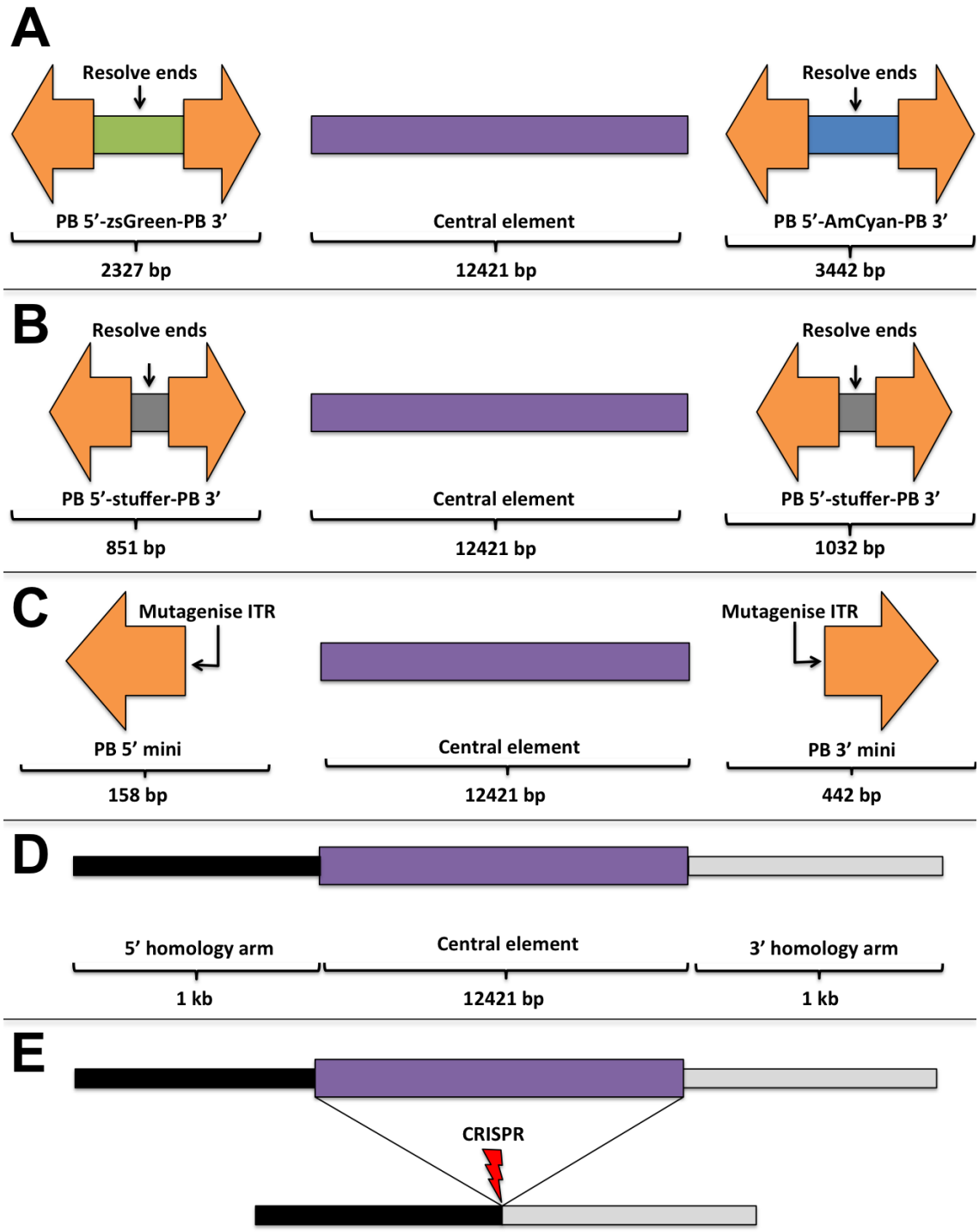


Figure 6.2. Optimisation of piggyBac transformation systems. (**A**) Current four-ended piggyBac (4-PB) vector with zsGreen and AmCyan markers to visualise end loss. (**B**) Proposed marker-free 4-PB vector system with small stuffers (100 bp) replacing markers (3.8 kb smaller). This could be applied as an alternative to CRISPR-mediated strategies. (**C**) Genomic immobilisation of 2-ended piggyBac constructs by CRISPR-mediated mutagenesis of inverted terminal repeat (ITR) sequences required for transposition at the 5' end, 3' end, or both ends. (**D-E**) Site-specific, piggyBac-free integration of an expression construct. (**D**) Expression construct diagram. (**E**) Induction of double-stranded breaks by CRISPR, to facilitate site-specific integration to genomic DNA. Lightning bolt indicates cleavage site.

6.2 Anti-zsGreen sgRNAs (SS1928-SS1931) with co-injected recombinant Cas9 protein mediate high-efficiency zsGreen mutagenesis

We optimised CRISPR parameters using the simplest experiment: indel-mediated knockout of a fluorescent marker sequence; site-specific integration by targeted recombination is size-dependent and comparably inefficient (Auer et al., 2014), (Gratz et al., 2014). Several formats exist for Cas9 (plasmid DNA, synthetic mRNA, recombinant protein, and transgenic lines expressing Cas9 in the germline) and for sgRNAs (plasmid DNA, synthetic mRNA, and genomically integrated U6 promoter-sgRNA transgenic lines), with promising reports for each (Bassett et al., 2013), (Gagnon et al., 2014), (Gratz et al., 2014). Germline-expressed Cas9 was apparently most efficient in *Drosophila* (Kondo and Ueda, 2013), (Ren et al., 2013), (Gratz et al., 2014). However, the practical difficulty of validating an equivalent Medfly strain (transgenesis, analysis of expression and functional validation) rationalised a parallel investigation of recombinant protein Cas9 (PNA Biotech CP01), which did not require the generation of transgenic lines. There was an additional advantage of microinjecting Cas9 as recombinant protein: it was directly transferrable to other species (no requirement to characterise efficient promoters and UTRs for each insect species, and clone expression constructs). Only *in vitro* transcribed sgRNAs were tested because several reports confirmed their functionality (Bassett et al., 2013), (Cho et al., 2013), (Jinek et al., 2013), (Yu et al., 2013), (Niu et al., 2014), (Zuris et al., 2014), and due to time availability, it was necessary to limit the scope of the study.

The standard *piggyBac* microinjection protocol was essentially used (**Chapter 2**), but we microinjected into the OX4014-A homozygous background (expresses AlMAct-dsRed2 and HR5IE1-zsGreen), rather than WT (**Figure 6.3**). Four anti-ZsGreen sgRNAs (SS1928-1931) were validated, to avoid inefficient sequences masking an efficient Cas9 effector. SS1928-SS1931 were assayed in vitro for their ability to target Cas9 protein to a zsGreen PCR amplicon. Activity was detected for SS1931, but not SS1928-1930. It was subsequently determined (after microinjection) that SS1928-1930 were accidentally synthesised with incorrect forward primers; we unintentionally generated sgRNAs that would bind to the opposite strand. These would not be expected to have cutting activity, because an nGG PAM motif was not present at the terminus of the binding site (Brouns et al., 2008).

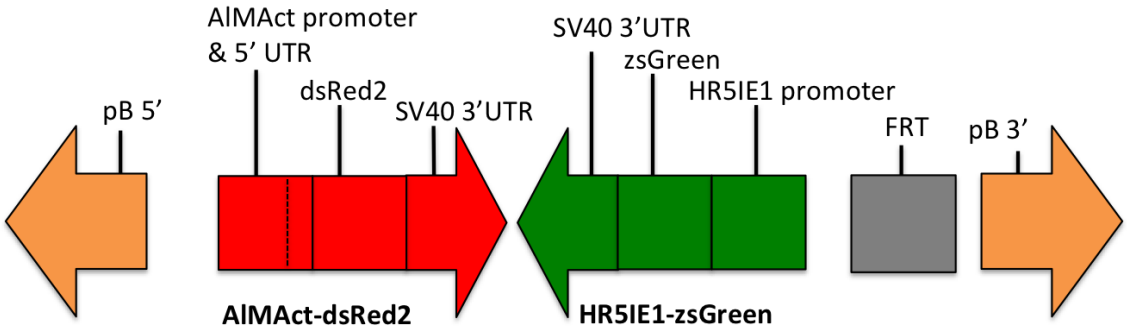


Figure 6.3. OX4014 expression construct diagram. Minimal construct featuring whole-body red fluorescent marker (AlMAct promoter-AlMAct 5'UTR-dsRed2-SV40 3'UTR) and green fluorescent marker (HR5IE1 promoter-*scraps* intron-nls-ZsGreen-nls-SV40 3'UTR). The FRT recombination site was not used. Dashes indicate promoter-5'UTR boundary.

The injection mix contained sgRNAs SS1928-1931 (250 ng/µl each) and Cas9 protein (1 µg/µl). Adult injection survival was less (19%) than the average results for injection of Medfly (25%) (Gregory et al., 2016). Surviving G_0 individuals were backcrossed to WT (**Tables 6.2-6.3**) and G_1 progeny screened for phenotypic reversion (presence of DsRed2, with loss of zsGreen).

Table 6.2 Injection logistics for Protein Cas9-SS1928-1931

Injection mix	Embryos	Larvae	Pupae	Adults	Knockouts
Protein Cas9-SS1928-1931	414	211 (51%)	119 (29%)	78 (19%)	33

Percentages in the "larvae", "pupae" and "adults" columns are survival rates.

Table 6.3 G_0 backcrosses to isolate zsGreen edits from Protein Cas9-SS1928-1931 injections

ProC9-SS1928-1931 A	**10 ♂ x 30 WT ♀**	**ProC9-SS1928-1931 F**	**5 ♀ x 5 WT ♂**
ProC9-SS1928-1931 B	10 ♀ x 10 WT ♂	ProC9-SS1928-1931 G	6 ♂ x 30 WT ♀
ProC9-SS1928-1931 C	**10 ♂ x 30 WT ♀**	ProC9-SS1928-1931 H	6 ♂ x 30 WT ♀
ProC9-SS1928-1931 D	**10 ♀ x 10 WT ♂**	ProC9-SS1928-1931 I	7 ♀ x 7 WT ♂
ProC9-SS1928-1931 E	**5 ♂ x 15 WT ♀**	**ProC9-SS1928-1931 J**	**9 ♀ x 9 WT ♂**

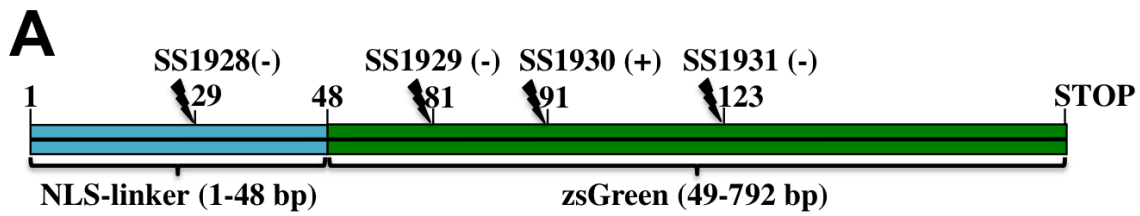
Underlined pools yielded edits.

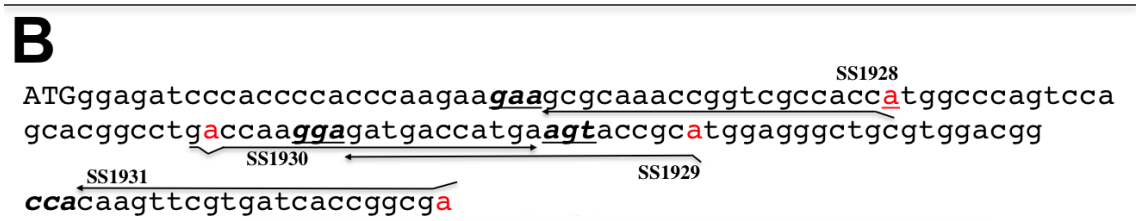
Protein Cas9-SS1928-SS1931 microinjections yielded 33 positives from 6/10 pools, all attributed to SS1931, as expected (**Table 6.4**). A PCR amplicon spanning the zsGreen target site was generated from each putatively mutagenised individual, ligated into the pJET vector, and sequenced as previously described. Three independent edits were detected in pool ProC9-SS1928-SS1931-C. Mutations in the other pools (A, D, E, F and J) were consistent between individuals of the same pool (**Figure 6.4**). Sequencing failed for one individual (D2). All events except ProC9-SS1928-SS1931-J were

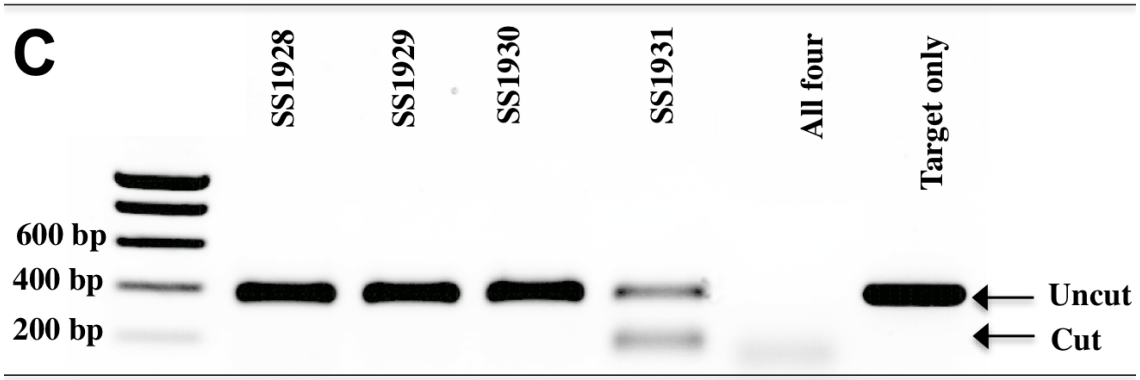
frameshifting indels. The results indicated that Cas9 mutagenesis leaves a distinct footprint, because (1) identical mutations were not found for individuals of different pools and (2) mutations for individuals of the same pool were generally identical (probably resulting from segregation of the edited chromosome to multiple cells during germline development). Efficiencies calculated were parental (pools with edited progeny/G_0 adult population) and progenital (proportion of G_1 progeny with a zsGreen knockout). The G_1 population was estimated by mean pupal weight from 30 replicates of 10 measured pupae (**Table 6.5**).

Table 6.4 Gene edits recovered from Protein Cas9-SS1928-SS1931 microinjections

Individual	Active sgRNA	Edit	Sequence change
A1	SS1931	2 bp deleted	F43→R43 & frameshift
C1		2 bp deleted	K42→N42 & frameshift
C2, C3, C5		2 bp deleted 4 bp inserted	H41→R41 & frameshift
C4		1 bp substituted 4 bp deleted	H41→R41 & frameshift
D1, D3-D10		4 bp inserted 5 bp deleted	K42→V42 & frameshift
E1-E6		2 bp deleted	F43→R43 & frameshift
F1-F2		8 bp deleted	K42→N42 & frameshift
J1-J9		21 bp deleted	In-frame deletion of H41-G49







D

ORIGINAL	ATG…[115 bp]…ACGGCCACAAGTTCGTGATCACCGGCGAGGG
A1	ATG…[115 bp]…ACGGCCACAAG--CGTGATCACCGGCGAGGG (2 bp del)
C1	ATG…[115 bp]…ACGGCCACAA--TCGTGATCACCGGCGAGGG (2 bp del)
C2,C3,C5	ATG…[115 bp]…ACGGTC----GTTCGTGATCACCGGCGAGGG (2 bp in/4 bp del)
C4	ATG…[115 bp]…ACGGTC----GTTCGTGATCACCGGCGAGGG (1 bp in/4 bp del)
D1,D3-D10	ATG…[115 bp]…ACGGCCACGTGA-CGTGATCACCGGCGAGGG (4 bp in/1 bp del)
E1-E6	ATG…[115 bp]…ACGGCCACAAG--CGTGATCACCGGCGAGGG (2 bp del)
F1-F2	ATG…[115 bp]…ACGGCCACAA--------TCACCGGCGAGGG (8 bp del)
J1-J9	ATG…[115 bp]…ACGGC----------GAGGG (21 bp del)

Figure 6.4. **Cas9-mediated mutagenesis of OX4014A hom-zsGreen (continued on next page)**. (**A-B**) sgRNA target positions (**A**) and target sequences (**B**) on zsGreen. PAM motifs indicated in bold italics. Lightning bolt indicates cleavage site. Mismatches at the 5' end of the sgRNA shown in red. Note that SS1928-SS1930 were synthesised incorrectly; we intended to target the reverse complement of these sequences. (**C**) *In vitro* validation of sgRNAs on zsGreen PCR product confirms functionality of SS1931 but not SS1928-1931. Expected cut sizes: SS1928 (106/258 bp), SS1929 (158/206 bp), SS1930 (168/196 bp), SS1931 (164/200 bp), all four (11 – 258 bp), uncut (364 bp). (**D**) Sequence alignments of modified individuals. PAM motif shown in bold italics. (**E**) Sequence traces of modified individual PC9-zsG-A1. PAM motif shown in bold italics.

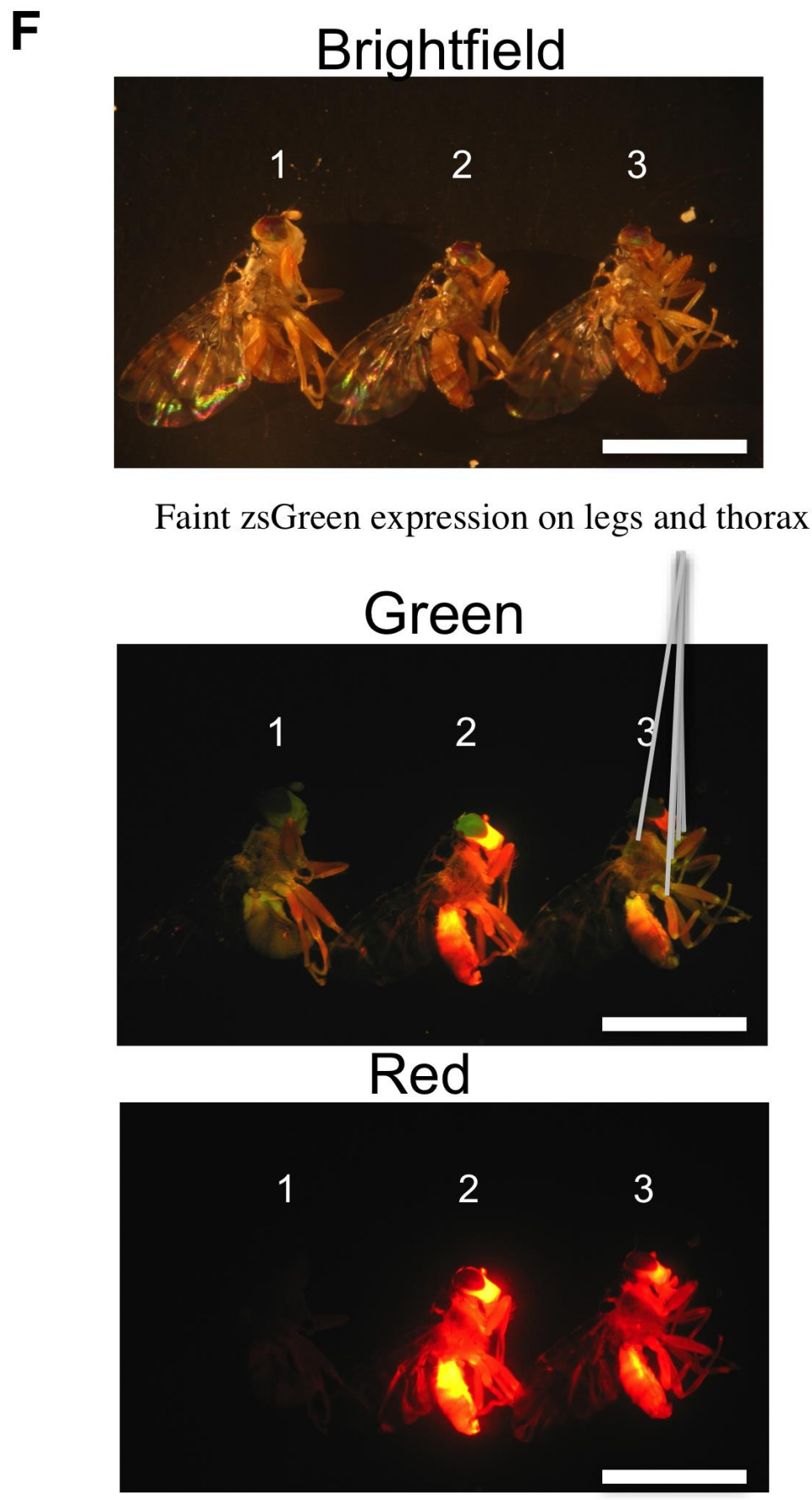


Figure 6.4. **Cas9-mediated mutagenesis of OX4014A hom-zsGreen**. (**F**) Images of WT [1], edited OX4014 [2, red only] and unedited OX4014 [3, red with faint zsGreen dots on legs and thorax]. zsGreen expression was readily visible by eye, but poorly visible on the imaging setup. Scale bars: 4 mm.

Table 6.5 Efficiency calculations for Protein Cas9-SS1928-SS1931 microinjections

	Estimating G_1 screening population			**Edits observed**		**Efficiency (%)**	
	Mass						
	Mean (mg)	Total (g)	Population	Pools	Progeny	Parental	Progenital
Protein Cas9 SS1928-SS1931	8.10	119.4	14747 ± 895	6	32	≥ 7.7	0.2 ± 0.01
OX5173 transgenesis	7.39	78.3	10590 ± 605	6	41	≥ 7.9	0.39 ± 0.02

Efficiency calculations: parental (pools with edited progeny/G_0 adult population) and progenital (proportion of G_1 progeny with zsGreen knockouts). Parental efficiencies were stated as greater than or equal to (≥), because it is possible that multiple G_0 individuals of the same pool demonstrated germline mutagenesis. The mean mass of 30 replicated measurements of 10 pupae were used to estimate the size of the screened G_1 population. Uncertainty given as standard deviation.

The calculation of progenital efficiency was particularly important, because high efficiency editing is required for non-laborious PCR screening of invisible mutations, such as *piggyBac* end removal or the loss of haplosufficient genes (genes required for male fertility that might be of commercial interest, are expected to be haplosufficient). For reference to the efficiency of *piggyBac* transgenesis, equivalent calculations were applied to the concurrently performed OX5173 (nanos-Cas9) transgenesis microinjection dataset. We next investigated whether promising reports of a germline-based delivery system in *D. melanogaster* could provide an equally valuable tool in Medfly.

6.3 Validation of a high efficiency germline (nanos-Cas9) expression system

6.3.1 Expression constructs used in the study

In *D. melanogaster*, *nanos*- or *vasa*-mediated germline Cas9 expression enhanced CRISPR editing efficiency, ostensibly from targeted maternal deposition of Cas9 to embryos (Kondo and Ueda, 2013), (Ren et al., 2013). *nanos* is conserved amongst diverse insects; it was therefore likely that the Medfly homologue (*Ccnanos*) would suitably regulate Cas9 (Curtis et al., 1995), (Lall et al., 2003). Sarah Scaife cloned a *piggyBac* vector (OX5134) with Ccnanos-Cas9 (*nanos* promoter-*nanos* 5'UTR-*S. pyogenes* Cas9-*nanos* 3'UTR) and an eye-specific transformation marker commonly used in *Drosophila*, but untested in Medfly (3xP3-zsGreen). Tissue-specific 3xP3 was chosen to minimise interference with other fluorescent markers (when crossed to other lines). Transformants were not recovered, indicating lack of 3xP3-ZsGreen function in Medfly. To confirm this, a double marker construct (OX5154) with (1) 3xP3-zsGreen and (2) previously validated HR5IE1-dsRed2 was generated (by Sarah Scaife). Transformants visibly expressed HR5IE1-DsRed2 but not 3xP3-ZsGreen, indicating 3xP3-zsGreen inactivity. Consequently, we selected HR5IE1-DsRed2 as the transformation marker and structured future experiments around the limitation that HR5IE1-dsRed2 is present in other constructs, and that its expression can be masked by the stronger AlMAct-dsRed2 transformation marker. A new vector (OX5173) was generated by modification of OX5134 (3xP3-green; Ccnanos-Cas9), substituting 3xP3-green for HR5IE1-dsRed2. These expression constructs are summarised in **Figure 6.5** with experiment-specific results below.

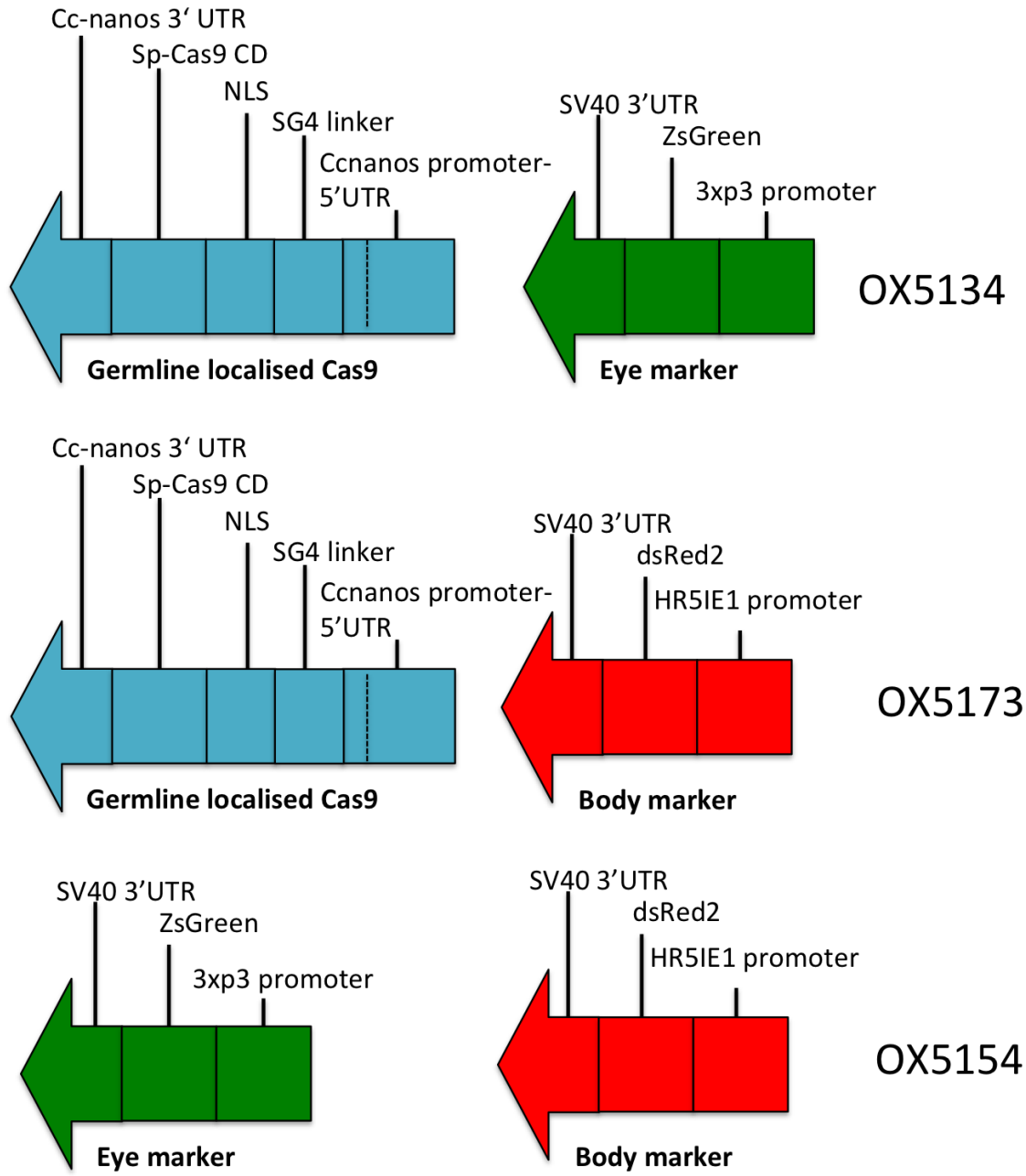


Figure 6.5. Schematic diagrams of nanos-Cas9 germline piggyBac expression constructs. OX5134: Nanos-Cas9 construct with 3xP3-zsGreen transformation marker (later proven non-functional). OX5173: Modification of OX5134 to include functional transformation marker: HR5IE1-dsRed2. OX5154: double marker construct (HR5IE1-dsRed2; 3xP3-zsGreen) used to confirm non-functionality of 3xP3-zsGreen. FRT recombination sites were vector artefacts (not experimentally applied). Dashes indicate promoter-5'UTR boundary. Note: piggyBac ends omitted from these two-ended vectors (full diagrams shown in Figure 2.2).

6.3.2 Failure to isolate OX5134 transgenic lines indicates inactivity of 3xP3-zsGreen in Medfly

Microinjection and backcrossing to WT (**Table 6.6**) were performed as previously described, using *piggyBac* helper OX3022 (300 ng/µl) and OX5134 (600 ng/µl). Transient 3xP3-zsGreen expression was not observed in G_0 pupae, the first indication that 3xP3-zsGreen was not visibly translated in Medfly. Adult survival (43%) was better than the average results for injection of Medfly (25%) (Gregory et al., 2016). Despite a large sample size (n = 359), transformants were not observed. Off-target Cas9 nuclease activity was not possible, because the sgRNAs required for activity were not present (Cradick et al., 2013). Therefore, it appeared that 3xP3-zsGreen was inactive, substantiated by unpublished data cited within a prior report (Schetelig and Handler, 2013).

Table 6.6 Microinjection logistics for OX5134

Construct	Embryos	Larvae	Pupae	Adults	Lines
OX5134	841	446 (53%)	363 (43%)	359 (43%)	0

Percentages in the "larvae", "pupae" and "adults" columns are survival rates.

6.3.3 Expression of HR5IE1-dsRed2 but not 3xP3-ZsGreen in OX5154 confirms lack of 3xP3 activity in Medfly

The eye-specific 3xP3 marker would be useful in other projects within Oxitec. Consequently, we decided to directly test for activity. Microinjection and backcrossing of a two-marker construct (OX5154) were conducted exactly as for OX5134 (**Tables 6.7-6.8**). Adult survival (51%) was higher than the average results for injection of Medfly (25%) (Gregory et al., 2016). In all five lines, HR5IE1-dsRed2 was visible, but not 3xP3-zsGreen. We investigated expression by RT-PCR to determine if the fragment had any activity. RNA was extracted from heads of flies (n=3) of 3 lines and used for cDNA synthesis with equivalent concentrations of RNA template for each sample (500 ng). OX5173E1 was tested in duplicate as a no-RT control. Non-quantitative RT-PCR was performed, monitoring expression of zsGreen and the constitutively expressed *Cc-RpP0* gene. Products were detected in OX5154 transgenic samples but not control, indicating transcription of 3xP3-zsGreen (**Figure 6.6**). Lack of fluorescence could be attributable to insufficient translation. We therefore modified the prior nanos-Cas9 vector (OX5134) to include a HR5IE1-dsRed2 transformation marker (OX5173).

Table 6.7 Microinjection logistics for OX5154

Construct	Embryos	Larvae	Pupae	Adults	Lines
OX5154	311	186 (60%)	169 (54%)	161 (51%)	5 (3%)

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (injection survivors yielding transgenic progeny).

Table 6.8 G0 backcrosses to establish OX5154 lines

OX5154A	**20 ♀ x 10 WT ♂**	OX5154B	20 ♀ x 10 WT ♂	**OX5154C**	**22 ♀ x 10 WT ♂**
OX5154D	**20 ♂ x 20 WT ♀**	**OX5154E**	**20 ♂ x 20 WT ♀**	OX5154F	20 ♂ x 20 WT ♀
OX5154G	20 ♂ x 20 WT ♀	**OX5154H**	**6 ♂ x 13 ♀**		

Underlined crosses yielded transgenic lines.

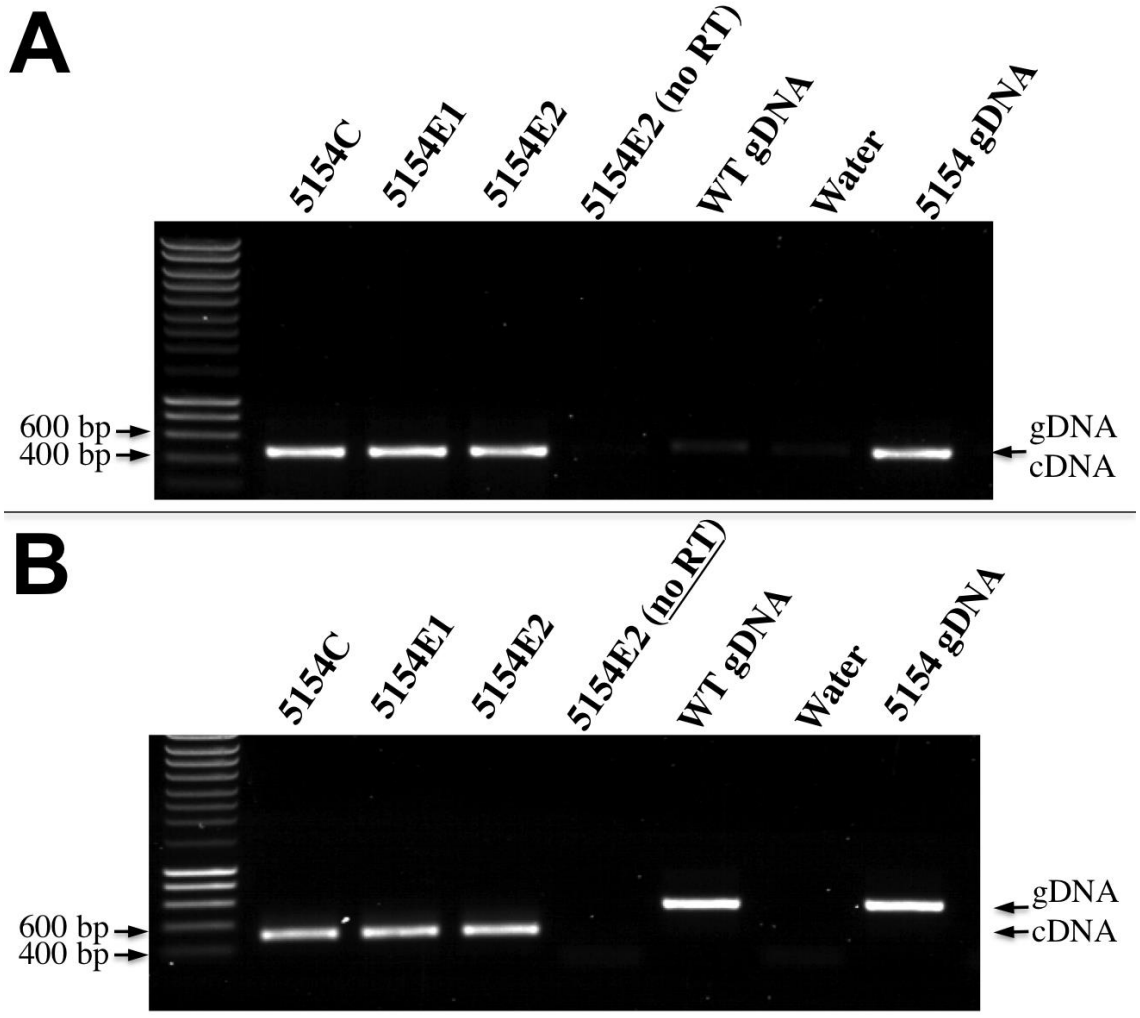


Figure 6.6. RT-PCR demonstrates that 3xP3-ZsGreen is transcriptionally active in three lines despite a failure to visualise the marker in pupae or adults. (A) 3xP3-ZsGreen reaction. (B) *Cc-RpP0* control reaction. Control templates were: OX5154E2-no RT (gDNA contamination); WT gDNA (control for non-specific priming), water (negative control), OX5154 gDNA (positive control). Expected product sizes: ZsGreen (cDNA & gDNA: 447 bp); *Cc-RpP0* (cDNA: 315 bp, gDNA: 542 bp).

6.3.4 OX5173 (nanos-Cas9) lines express Cas9 in the ovaries and embryonic surface (maternal deposition)

Microinjection and backcrossing to WT was conducted exactly as for OX5134/OX5154 (**Tables 6.9-6.10**). Adult survival (51%) was higher than the average results for injection of Medfly (25%) (Gregory et al., 2016). Mendelian assessment of transgenic lines was performed as previously described (**Table 6.11**). Eleven lines (OX5173C, D, E1-E5, G1-G2, I, L) were isolated from 6 pools (OX5173E and OX5173G provided five and two distinct lines). OX5173C was randomly discarded to reduce line number to ten. All lines except OX5173E5 were single insertions. The insertion sites of OX5173E1, E4, E5 & G2 were not determined (female crosses). OX5173E2 and E3 were Y-linked and OX5173L was X-linked. All other lines (OX5173D, G1 & I) were autosomal insertions. Double insertions require segregation to determine if one or both insertions are associated with active transgenic expression. Y-linked transgenes cannot be propagated in females; the female lineage is required for maternal deposition of nanos-Cas9. Consequently, we selected single autosomal insertion lines (OX5173D, E1, E4, G1, G2, I) and an X-linked insertion (OX5173L) to confirm Cas9 expression by RT-PCR.

Table 6.9 Injection logistics for OX5173

Embryos	Larvae	Pupae	Adults	Lines
279	211 (78%)	149 (53%)	139 (50%)	11 (8%)

Percentages in the "larvae", "pupae" and "adults" columns are survival rates. The percentage in the "lines" column is the transformation efficiency (injection survivors yielding transgenic progeny).

Table 6.10 G0 backcrosses to establish OX5173 lines

OX5173A	10 ♂ x 30 WT ♀	OX5173B	10 ♂ x 30 WT ♀	**OX5173C**	**10 ♂ x 30 WT ♀**
OX5173D	**10 ♂ x 30 WT ♀**	**OX5173E**	**10 ♂ x 30 WT ♀**	OX5173F	20 ♀ x 10 WT ♂
OX5173G	**17 ♀ x 10 WT ♂**	OX5173H	10 ♂ x 30 WT ♀	**OX5173I**	**8 ♂ x 24 WT ♀**
OX5173J	8 ♂ x 24 WT ♀	OX5173K	13 ♀ x 10 WT ♂	**OX5173L**	**13 ♀ x 10 WT ♂**

Underlined pools yielded transgenics.

Table 6.11 Mendelian analysis of OX5173 transgenic lines

Line	Sex	Copy number		Sex-linkage		Summary		Cas9 cDNA in ovaries
		Transgenic (%)	n	Sex ratio (M/F)	n	Copies	Location	
OX5173D	♂	55	121	1.1	29	1	Autosome	Yes
OX5173E2	♂	50	239	Male-only	23	1	**Y**	
OX5173E3	♂	50	58	Male-only	60	1	**Y**	
OX5173G1	♂	49	274	0.9	54	1	Autosome	Yes
OX5173I	♂	53	134	0.7	37	1	Autosome	Yes
OX5173L	♂	39	31	Female-only	8	1	**X**	No
OX5173E1	♀	63	49	1.0	30	1		No
OX5173E4	♀	62	45	0.6	27	1		Yes
OX5173E5	♀	**77**	39	2.0	18	**2**		
OX5173G2	♀	48	97	0.9	27	1		Yes

Sex-linkage is only apparent from crosses in which the transgenic parent is male. Therefore, the sex-linkage of female transgenic crosses was not determined (OX5173E1, E4, E5 & G2).

Seven lines (OX5173D, E1, E4, G1, G2, I and L) were assessed for Cas9 transcription in ovaries and embryos by RT-PCR, to select nanos-Cas9 lines with high expression for enhanced gene editing. Five virgin heterozygous females from each of 7 OX5173 lines and WT (control) were crossed to WT males (1:1) and allowed to mate (7 days). This was performed because mating can affect the expression of ovarian genes (McGraw et al., 2004), (McGraw et al., 2008). Ovaries were dissected and RNA extracted, prior to cDNA synthesis with equalised RNA template (500 ng RNA per sample). OX5173E1 was duplicated as a no-RT control. RT-PCR amplicons were generated for Cas9 and the constitutively expressed control gene *Cc-RpP0*. Reactions were duplicated for semi-quantitative assessment at 25, 30, 35 and 40 cycles. Cas9 was detected in the ovaries of 5 lines (OX5173D, E4, G1, G2 and I). After 35 cycles, OX5173D and OX5173G2 demonstrated the highest levels of Cas9 expression (**Figure 6.7**).

As ovarian expression was not necessarily correlated with maternal deposition to embryos, further RT-PCR analysis was performed on eggs of OX5173 females. Crosses were 50 heterozygous OX5173 or WT (control) females to 50 WT males. Eggs from a 4 hour egg collection (day 5) were homogenised by freezing and pestle crushing. Greater input RNA (750 ng) was used because reduced expression relative to ovaries was possible; the analysis was otherwise identical. The findings of the ovarian study were generally recapitualated (**Figure 6.7**). Surprisingly, ovarian expression was not always associated with maternal Cas9 deposition. Cas9 mRNA was detected in ovaries, but not eggs, from OX5173I. OX5173D and G1 were selected for future analysis on the basis of highest apparent expression (OX5173G1 and G2 were similar).

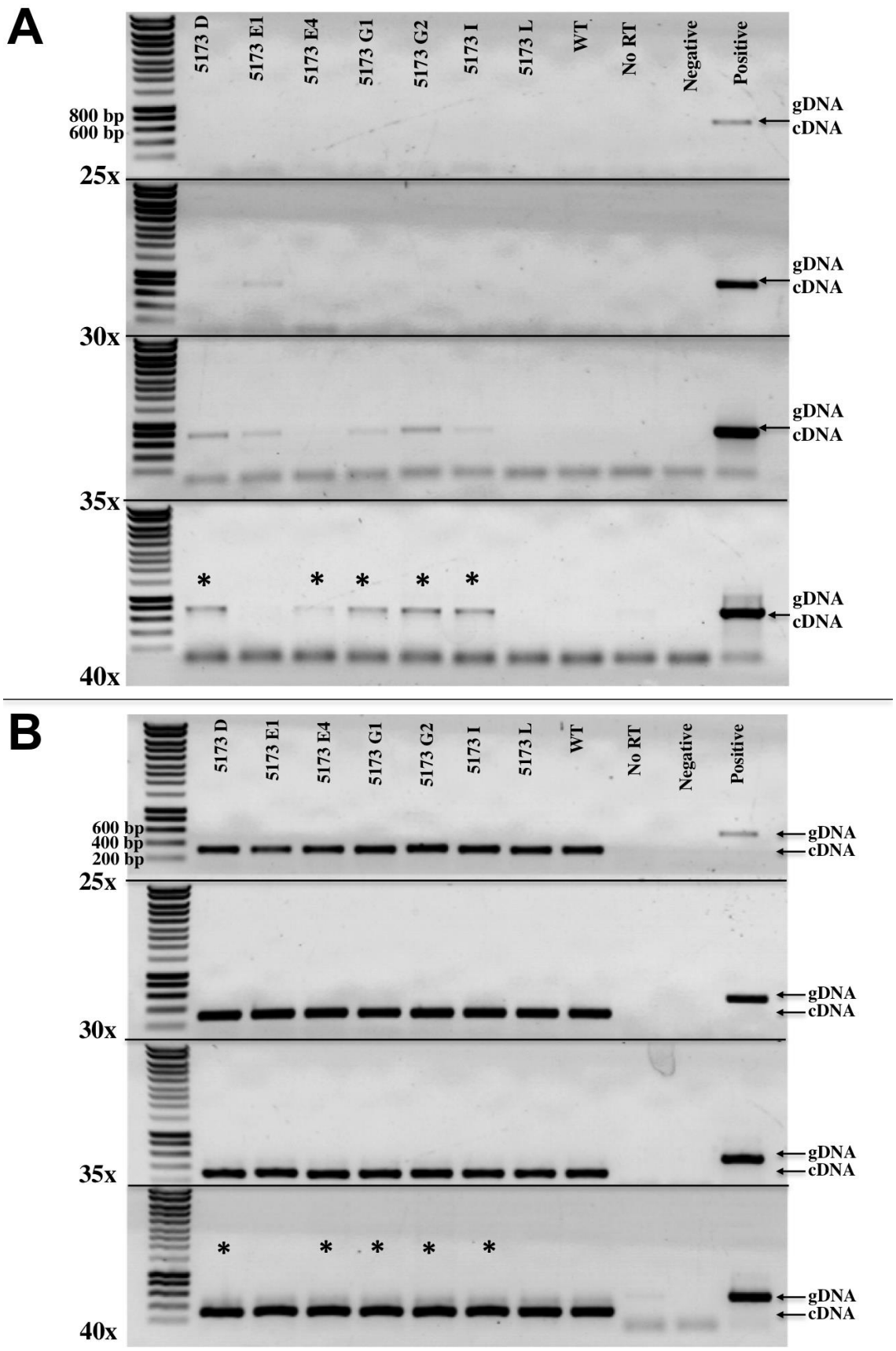


Figure 6.7. Semi-quantitative RT-PCR (25, 30, 35 & 40 cycles) confirms nanos-driven Cas9 expression in ovaries and eggs of OX5173 lines (continued on next page). (**A-B**) Cas9 is expressed in ovaries of 5/7 OX5173 lines (*). (**A**) Cas9 reaction. (**B**) *Cc-RpP0* control reaction. Expression was strongest in OX5173D & G2. RT-PCR controls were no reverse transcriptase (gDNA contamination), water (negative) and OX5173D gDNA (positive). Expected product sizes: Cas9 (cDNA/gDNA: 751 bp); *Cc-RpP0* (cDNA: 315 bp, gDNA: 542 bp).

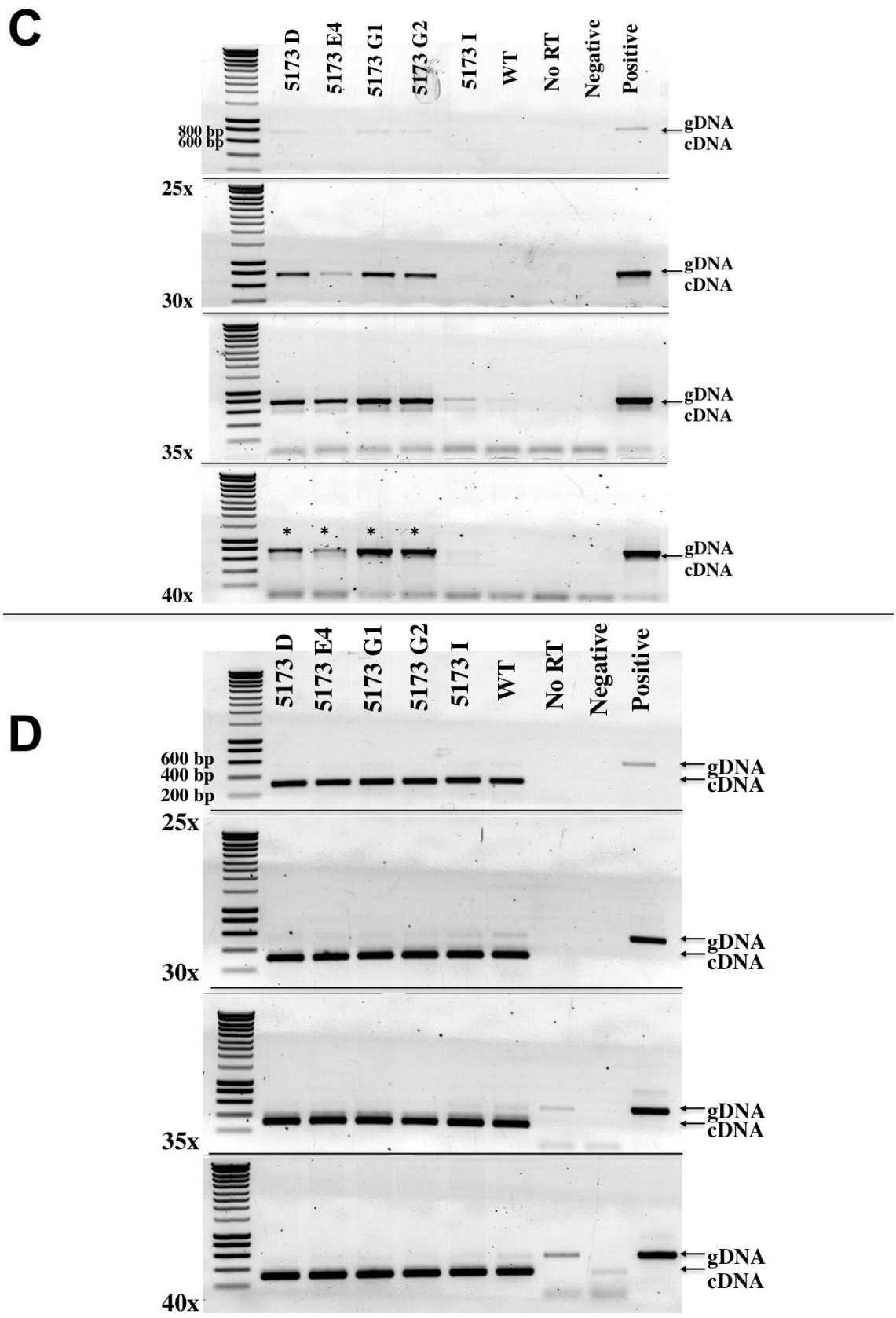


Figure 6.7. Semi-quantitative RT-PCR (25, 30, 35 & 40 cycles) confirms nanos-driven Cas9 expression in ovaries and eggs of OX5173 lines (continued on next page). (**C-D**) Cas9 mRNA is maternally deposited to eggs of 4/5 OX5173 lines (*) with ovarian Cas9 expression. (**C**) Cas9 reaction. (**D**) *Cc-RpP0* control reaction. The lines with strongest apparent expression (OX5173D and G1) were selected for future analysis. Mild contamination with gDNA was evident in the no-RT reaction. RT-PCR controls were no reverse transcriptase (gDNA contamination), water (negative) and OX5173D gDNA (positive). Expected product sizes: Cas9 (cDNA/gDNA: 751 bp); *Cc-RpP0* (cDNA: 315 bp, gDNA: 542 bp).

Figure 6.7. Semi-quantitative RT-PCR (25, 30, 35 & 40 cycles) confirms nanos-driven Cas9 expression in ovaries and eggs of OX5173 lines. (**E**) Images of transgenic OX5173E1-E5 lines.

6.3.5 Microinjection of anti-dsRed2 sgRNAs (SS2045 & SS2047) into a nanos-Cas9 expressing strain (OX5173D) mediates high-efficiency dsRed2 mutagenesis

We validated the germline-expressed nanos-Cas9 activity of OX5173 lines, again by mutagenesis of marker sequences. Difficulty screening zsGreen loss in the protein injection experiment (ProCas9-SS1928-SS1931) was considered to potentially underestimate efficiency (extremely early screening was required, HR5IE1-zsGreen is less visible than AlMAct-dsRed2). Consequently, we screened for DsRed2 mutagenesis. Microinjection and WT backcrosses were performed essentially as described for the protein Cas9-SS1928-SS1931 microinjections (**Tables 6.12-6.13**). OX5173D and G1 were assessed concurrently. The injection background was F_1 progeny of a homozygous OX4014 male (n=125) to heterozygous OX5173 female (n=250) cross. Buffered injection mixes contained activity-validated sgRNAs SS2045 & SS2047 (500 ng/µl each, provided by Justine Braguy and Sarah Scaife). Adult injection survival (OX5173D: 19%; OX5173G1: 27%) was similar to the average results for injection of Medfly (25%) (Gregory et al., 2016).

Table 6.12 Injection logistics for OX5173-SS2045-SS2047 injections

Line	Embryos	Larvae	Pupae	Adults	Knockouts
OX5173D	565	257 (45%)	183 (32%)	106 (19%)	511
OX5173G1	648	253 (39%)	209 (32%)	178 (27%)	0

Percentages in the "larvae", "pupae" and "adults" columns are survival rates.

Table 6.13 G_0 backcrosses to isolate OX5173-SS2045-SS2047 dsRed2 edits

OX5173D-SS2045-SS2047-A	5 ♂ x 15 WT ♀	**OX5173D-SS2045-SS2047-B**	**5 ♂ x 15 WT ♀**
OX5173D-SS2045-SS2047-C	**5 ♂ x 15 WT ♀**	OX5173D-SS2045-SS2047-D	5 ♂ x 15 WT ♀
OX5173D-SS2045-SS2047-E	**5 ♂ x 15 WT ♀**	**OX5173D-SS2045-SS2047-F**	**5 ♀ x 5 WT ♂**
OX5173D-SS2045-SS2047-G	**5 ♀ x 5 WT ♂**	**OX5173D-SS2045-SS2047-H**	**5 ♀ x 5 WT ♂**
OX5173D-SS2045-SS2047-I	**5 ♀ x 5 WT ♂**	**OX5173D-SS2045-SS2047-J**	**5 ♀ x 5 WT ♂**
OX5173D-SS2045-SS2047-K	**5 ♂ x 15 WT ♀**	OX5173D-SS2045-SS2047-L	5 ♂ x 15 WT ♀
OX5173D-SS2045-SS2047-M	5 ♀ x 5 WT ♂	OX5173D-SS2045-SS2047-N	5 ♀ x 5 WT ♂
OX5173D-SS2045-SS2047-O	**25 ♂ x 75 WT ♀**	**OX5173D-SS2045-SS2047-P**	**25 ♀ x 25 WT ♂**
OX5173G1-SS2045-SS2047-A	5 ♂ x 15 WT ♀	OX5173G1-SS2045-SS2047-B	5 ♂ x 15 WT ♀
OX5173G1-SS2045-SS2047-C	5 ♂ x 15 WT ♀	OX5173G1-SS2045-SS2047-D	5 ♂ x 15 WT ♀
OX5173G1-SS2045-SS2047-E	5 ♂ x 15 WT ♀	OX5173G1-SS2045-SS2047-F	5 ♀ x 5 WT ♂
OX5173G1-SS2045-SS2047-G	5 ♀ x 5 WT ♂	OX5173G1-SS2045-SS2047-H	5 ♀ x 5 WT ♂
OX5173G1-SS2045-SS2047-I	5 ♀ x 5 WT ♂	OX5173G1-SS2045-SS2047-J	5 ♀ x 5 WT ♂
OX5173G1-SS2045-SS2047-K	5 ♀ x 5 WT ♂	OX5173G1-SS2045-SS2047-L	5 ♀ x 5 WT ♂
OX5173G1-SS2045-SS2047-M	5 ♂ x 15 WT ♀	OX5173G1-SS2045-SS2047-N	5 ♂ x 15 WT ♀
OX5173G1-SS2045-SS2047-O	50 ♂ x 150 WT ♀	OX5173G1-SS2045-SS2047-P	50 ♀ x 50 WT ♂

Underlined pools yielded edits.

For each genotype (OX5173D or OX5173G1), backcrosses of F_1 microinjection survivors (seven pools per sex) were initiated with identical populations (n=5) to facilitate comparisons of efficiency. Surplus individuals were crossed in two sex-specific pools. Relative to the prior experiment (Protein Cas9-SS1928-SS1931, **Section 6.2**), we reduced and equalised pool size to (1) directly compare OX5173D/G1 and (2) limit underestimation of efficiency (matching unique edits to independent parents of a pool is impossible). Crosses of single individuals were not performed, because we were not yet certain that either OX5173 line could mediate mutagenesis. F_2 progeny were screened for a HR5IE1-zsGreen positive, AlMAct-dsRed2 negative phenotype (HR5IE1-DsRed2 [OX5173] is readily distinguished from AlMAct-DsRed2 [OX4014]). The efficiency of editing was calculated, largely as described for the Protein Cas9-SS1928-SS1931 experiment (**Table 6.14)**.

Table 6.14 Efficiency calculations for OX5173D-SS2045-SS2047 microinjections

	Estimating F_2 screening population			**Edits observed**		**Efficiency (%)**	
	Mass						
	Mean (mg)	Total (g)	Population	Pools	Progeny	Parental	Progenital
OX5173D SS2045-SS2047	8.40	161.1	19168 ± 1593	9	511	≥ 13	2.7 ± 0.22
OX5173G1 SS2045-SS2047	8.37	163.8	19572 ± 1677				
OX5173 transgenesis	7.39	78.3	10590 ± 605	6	41	≥ 7.9	0.39 ± 0.02

The F_2 population was estimated by mean pupal weight from 50 replicates of 10 measured pupae, except for OX5173 transgenesis (30 replicates of 10 pupae, scored concurrently with the Protein Cas9-SS1928-SS1931 microinjection dataset). Uncertainty given as standard deviation. It was not calculated for parental efficiency, because these measures were not possible to replicate.

Mutagenesis was detected for F_2 progeny of OX5173D (n = 511) but not for OX5173G1, despite a larger F_1 cohort (**Table 6.15**). The parental efficiency was most likely underestimated because the presence of different edits within the same pool, indicated possible origin from independent parents (however, it was not possible to exclude the possibility of origin from the same parent). As we crossed injection survivors in pools of five, it was not possible to measure parental efficiency as greater than 20% (ie. 100/5). DsRed2 PCR amplicons were generated from two positives from each pool (n = 22 [11 x 2]) and the unmodified OX4014 background, prior to direct sequencing with internal primers. Fifteen independent events were confirmed. Thirteen were indels from a single sgRNA (SS2045: n=12, SS2047: n=1) and two were deletions that spanned the region between SS2045-SS2047. Sequencing data from 5 events is presented (**Figure 6.8**). The inability of OX5173G1 to mediate editing was unexpected,

as the RT-PCR indicated expression in ovaries and eggs. At this point, time expired to perform further experiments. Homozygosis of OX5173D was initiated and future recommendations for study are discussed subsequently.

Table 6.15 Gene edits recovered from OX5173D-SS2045-SS2047 microinjections

Individual	Active sgRNA	Edit	Sequence change
B1/B2	SS2045	32 nt deleted	Frameshift at A2; 10 codons deleted
C1	SS2045	1 nt inserted 10 nt deleted	Frameshift at M1; 3 codons deleted
C2	SS2045	13 nt deleted	Frameshift at A2; 4 codons deleted
E1/E2	SS2045	13 nt deleted	Frameshift at S4; 4 codons deleted
F1	SS2045/SS2047	285 nt deleted	In-frame deletion of S4-N98
F2	SS2045	3 nt deleted	Non-sense mutation at S4
G1/G2	SS2045	34 nt deleted	Frameshift at A2; 11 codons deleted
H1/H2	SS2045	16 nt deleted	Frameshift at M1; 5 codons deleted
I1/I2	SS2045	30 nt deleted	In-frame deletion of S4-R13
J1/J2	SS2045	2 nt deleted	Frameshift at S4
K1/K2	SS2047	14 nt deleted	Frameshift at R95; 4 codons deleted
O1	SS2045/SS2047	284 nt deleted	Frameshift at S4; 94 codons deleted
O2	SS2045	11 nt deleted	Frameshift at A2; 3 codons deleted
P1	SS2045	11 nt deleted	Frameshift at S4; 3 codons deleted
P2	SS2045	13 nt deleted	Frameshift at S4; 4 codons deleted

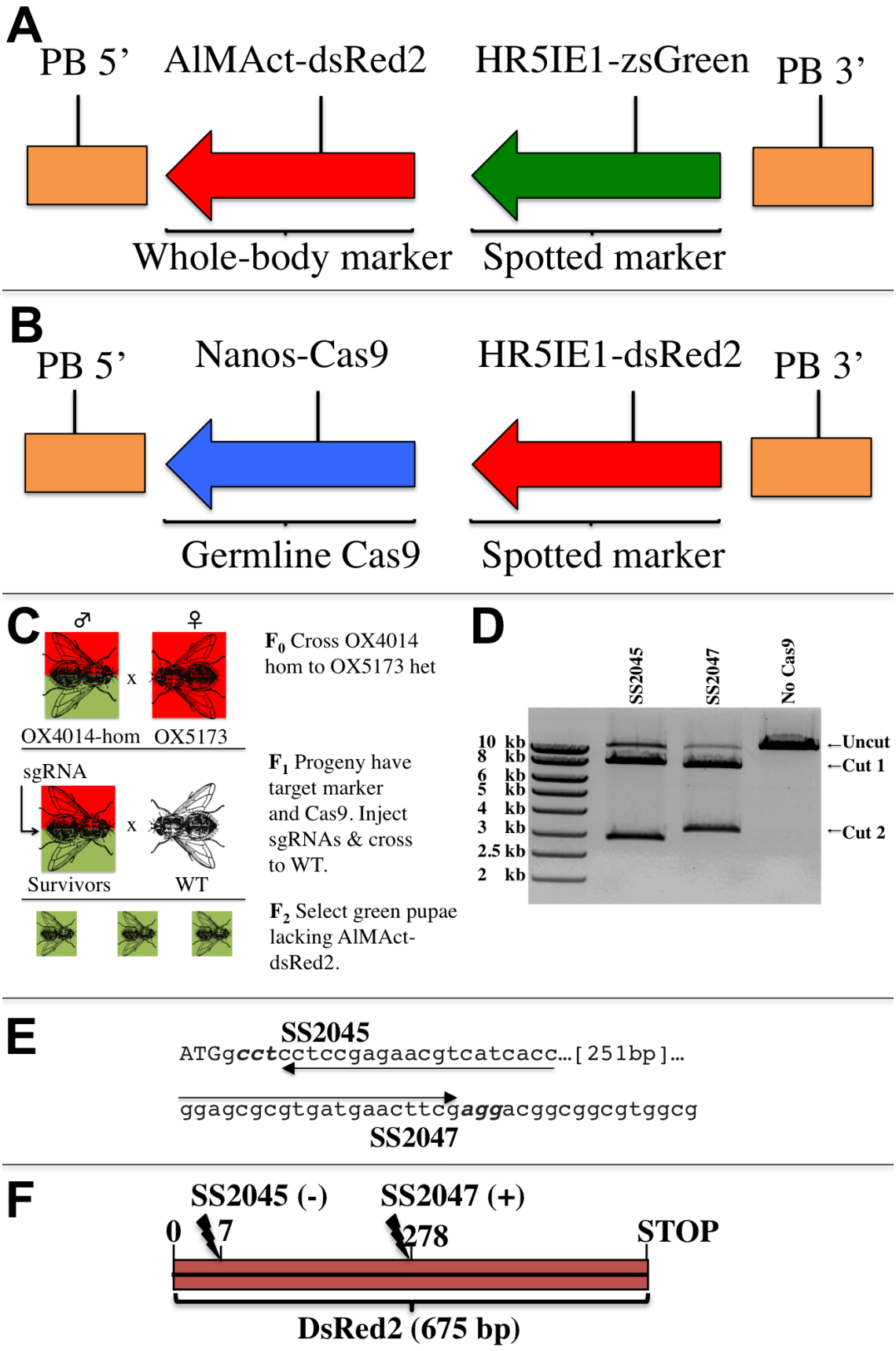
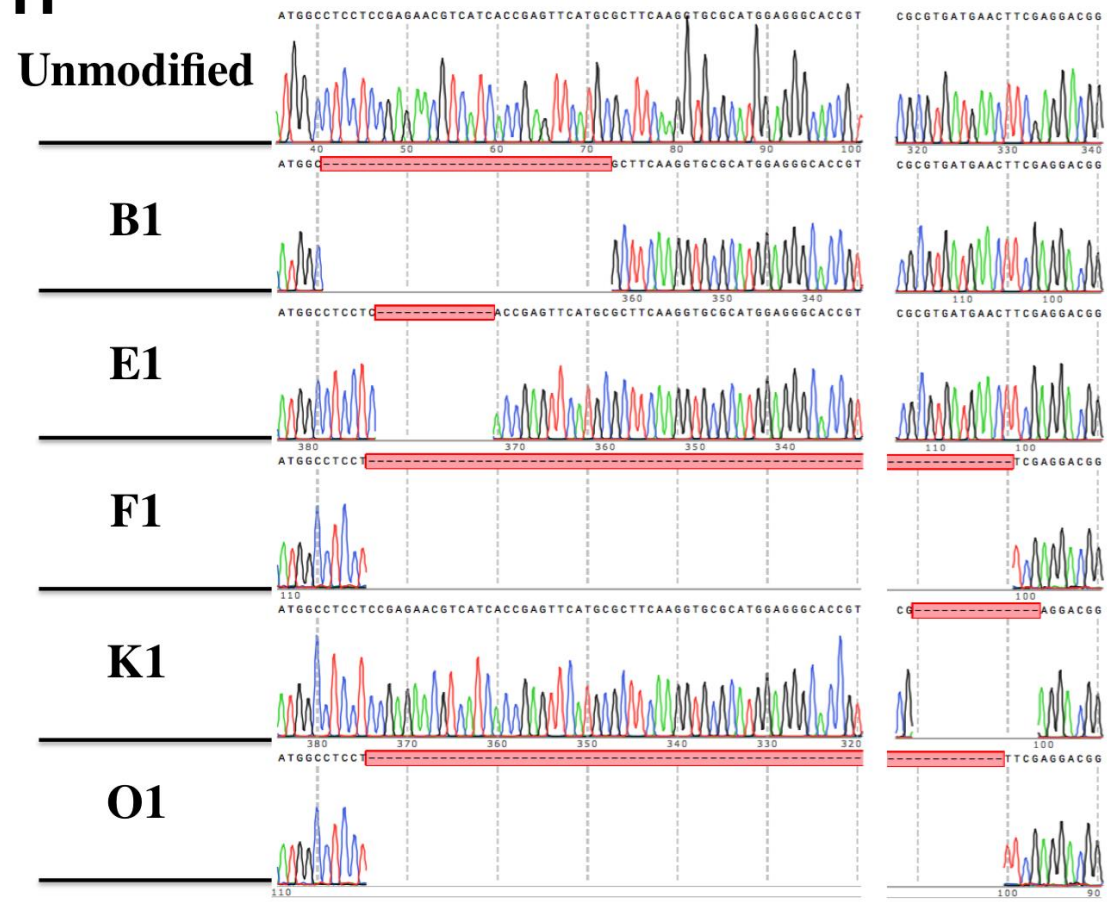


Figure 6.8. Germline Cas9 expression (OX5173D) mediates high-efficiency mutagenesis of dsRed2 (continued on next page). (A) OX4014 (marker line) vector. (B) OX5173 (nanos-Cas9) vector. (C) Experimental overview of microinjections and crosses. (D) Validation of SS2045 & SS2047 activity on SacI-OX4014 DNA (expected sizes: SS2045 = 2849 & 7779 bp; SS2047 = 3121 & 7507 bp; uncut = 10628 bp). (E-F) SS2045 & SS2047 target sequences (E) and target positions (F) on dsRed2. PAM motif in bold italics. Lightning bolt indicates cleavage site.

G

```
ORIGINAL   ATGGCCTCCTCCGAGAACGTCATCACCGAGTTCATGCGC…[243 bp]…CGCGTGATGAACTTCGAGGA…[376 bp]…STOP
B1         ATGGC----------------------------------GC…[243 bp]…CGCGTGATGAACTTCGAGGA…[376 bp]…STOP (32 bp del)
E1         ATGGCCTCCTC-----------ACCGAGTTCATGCGC…[243 bp]…CGCGTGATGAACTTCGAGGA…[376 bp]…STOP (13 bp del)
F1         ATGGCCTCCT-------------------------------------------------TCGAGGA…[376 bp]…STOP (285 bp del)
K1         ATGGCCTCCTCCGAGAACGTCATCACCGAGTTCATGCGC…[243 bp]…CG-----------AGGA…[376 bp]…STOP (14 bp del)
O1         ATGGCCTCCT-----------------------------------------------TTCGAGGA…[376 bp]…STOP (284 bp del)
```

H



I

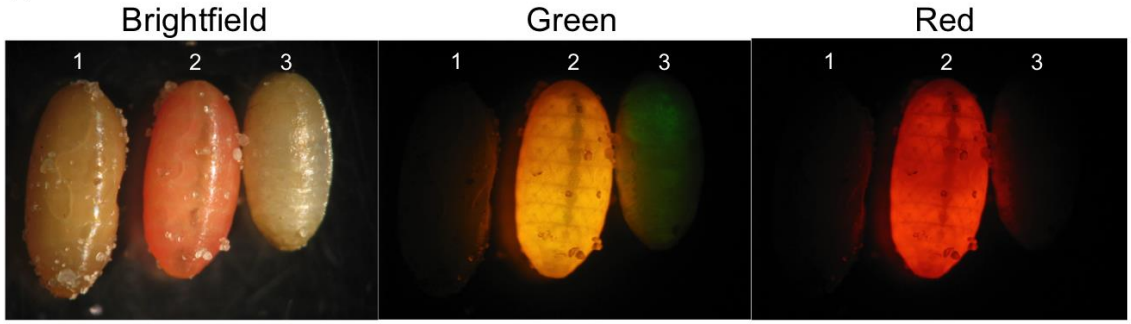
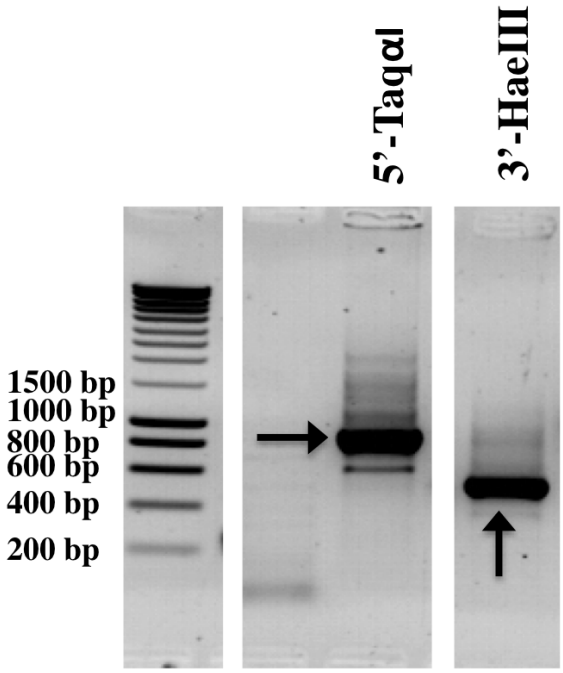


Figure 6.8. Germline Cas9 expression (OX5173D) mediates high-efficiency mutagenesis of dsRed2. (G-H) Sequence alignments (G) and sequence traces (H) of edited individuals. (I) Images of WT [1], unedited OX4014 [2, red masking faint green dots] and edited OX4014 [3, green dots]. Scale bars: 4 mm.

6.3.6 Isolation of genomic sequence flanking the OX5173D insertion site

The flanking genomic sequence of OX5173D would prove useful for PCR-mediated homozygosis, and to characterise a transgenic docking site with favourable expression of maternally deposited transgenes (for future site-specific integration). Inverse PCR was successful for TaqαI (5' end) and HhaI (3' end) digestions. The dominant bands were gel purified, cloned into the pJET vector and sequenced. Contiguous fragments spanning the insertion site and genomic DNA were isolated (**Figure 6.9**) at the 5' (778 bp) and 3' ends (914 bp).

A



B **OX5173D 5' flanking sequence**

GCGATGACGAGCTTGTTGGTGAGGATTCTGACAGTGAAATATCAGATCACGTAAGTGAAGATGACG
TCCAGAGCGATACAGAAGAAGCGTTTATAGATGAGGTACATGAAGTGCAGCCAACGTCAAGCGGTA
GTGAAATATTAGACGAACGAAATGTTATTGAACAACCAGGTTCTTCATTGGCTTCTAACAGGATCT
TGACCTTGCCACAGAGGACTATCAGAGGTAAGAATAAACATTGTTGGTCAACTTCAAAGTCCACGA
GGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGATCTCAAAGAGGTCCGACGCGTATGTGCCGCA
ATATATATGACCCACTTTTATGCTTCAAACTATTTTTTACTGATGAGATAATTTCGGAAATTGTAA
AATGGACAAATGCTGAGATATCATTGAAACGTCGGGAATCTATGACAGGTGCTACATTTCGTGACA
CGAATGAAGATGAAATCTATGCTTTCTTTGGTATTCTGGTAATGACAGCAGTGAGAAAAGATAACC
ACATGTCCACAGATGACCTCTTTGATCGATTGGGAGCTCCAGTTCGCCTACATCCTATGCTTCCCT
TTCATTAGAATTTCTCAGACTGGCGCAGAGTTCACCGCATAAAGCCAGTATACAAGTACGCCAATT
CACCACTTTGGCTGCTACATTAGTATGATCCGGTCTGGAAACCATCTGCAATGCTTCAACTACCCA
TCTCACATACCTCCCAGTCATAGCAACCTATCTTCTGATAGCGAAATGTCAA**TTAA**

OX5173D 3' flanking sequence

TTAAAAGCCGTCACCGCTTGTCGATGCCGCAGAAAATACTCGTACTTGTGTGTTCTCTTTCAGTCG
GTTGGTACGGCTGTGGGATACCATGTAAGATGGATAGTTGCAGTCACAAGGATTTTTGCATACAAA
ATTCGTGTATTTCTTGGATCGGCCAGGCTCTCAAGCTCTTATGCTACGCTGTCGGCCTCTCTCTTT
TGTTTGTCATTTCCAACAGTTAAGGAAAGCAAATTAAGGAAAATGCTTGGAAACTTTAATAAACTT
GTATTGGAAACTATGAATGAAATATTATTTAGAATTTTTGTTTTTAGGTTAGGTTGAACTGGCTGA
ATCATAGAAAAGTAATATTTTTATGTTCTAGTATTTAATAAATAATAGAAAGCATTTACATACGCT
CATACATATGTATATATGCTTATACTTTTATATTGGACTAACACTTCTTAGCCTCACAATTTTAAA
ATCCCCTAAGCCGCTTAAATTATATGCAGCCATTTGCCTCCATTGGCCAGTGCACAGCTACAGGTA
CCCATACACGCCCATGTAGTTGTTACATAAGTGTTATGCAATGCAAAGGTTAAACAATGAAACTAT
TCAGTCTTATTTTTTTTTAACCGGAATTCAGTCAGTCAGTTTTATGAACTCTCTTAATCCTCATAA
TCGCCTACATAATCAGCCCTAATTTGAGCACTTCATGAATTTCTTTGTACAAACATACATACAGAT
GAATGTACATATGTATGTATATGTTTGTATTTATACATGTCGTTCTTCCAACACTCGGCGCGCGAT
AATATCTCTAATATTTTGCCAAATGAAGTGCCTGGTACATCAGATGACAGTACTGAAGAGCCAGTA
ATGAAAATACGTACTTACTGTACTTACTGCCCCTCTAAAATAAGGCGAAAGGCAAATGCA

Figure 6.9. Isolation of OX5173D flanking sequence and preliminary work for homozygosis. (A) Inverse PCR isolation of 5'-TaqαI-OX5173D and 3'HhaI-OX5173D flanking sequence. Block arrows indicate the band excised from the gel. (B) OX5173D 5' and 3' flanking sequences with TTAA termini.

6.4 Conclusions

CRISPR technology was applied to mediate highly efficient gene editing of Medfly, and a protocol for modifying Medfly or related insects established. Although the microinjection of recombinant Cas9 protein was functional, the high efficiency observed for germline-expressed Cas9 (OX5173D) supports the creation of equivalent strains for other insects. This would be particularly important for site-specific integration, which is less efficient than the generation of indels (Gratz et al., 2014). Germline modification was induced in ≥ 13% of injection survivors (parental efficiency), with heritable mutations in 2.7% of screened progeny (progenital efficiency). This was substantially less than the average parental efficiency (100%) and progenital (60%) efficiency, reported in a *D. melanogaster* study (Kondo and Ueda, 2013). However, there were substantial differences in the methodology of the two studies, which must be considered.

First, we crossed individuals in pools of five, as the ability of the OX5173 lines to mediate mutagenesis had not yet been confirmed. Therefore, it would not be possible to report a parental efficiency higher than 20%. The possibility of underestimation is substantiated by the frequent observation of different mutations in G_2 progeny originating from the same pool; in the recombinant Cas9 protein experiment, almost all G_2 progeny of the same pool had the same mutation. This suggests, but does not confirm, that multiple G_1 individuals had germline mutations. It would be necessary to repeat the analysis with crosses of single individuals, to fully determine this efficiency. Furthermore, flies in the published study expressed sgRNA in the germline (U6 promoter-sgRNA); sgRNAs were microinjected in our study. We observed a striking improvement in efficiency between the strain expressing Cas9 in the germline (OX5173), relative to the microinjection of Cas9 as recombinant protein. Therefore, it would be expected that the efficiencies would be improved by germline expression of sgRNA, relative to microinjection of *in vitro* transcribed sgRNAs. To mediate this, we isolated and cloned the U6 promoter sequences from Medfly (**Appendix, Figure 8.2**). Unfortunately, there was insufficient time to investigate their ability to mediate efficient germline mutagenesis.

Though preliminary, a framework is established for more technical experiments. Two applications were particularly relevant commercially: transposon immobilisation by mutagenesis and site-specific construct integration. CRISPR-mediated

immobilisation of two-ended constructs would allow for a substantial reduction in the size of expression constructs (3.8 kb), relative to the four-ended *piggyBac* system with three fluorescent markers applied in prior study (**Chapter 5**). As previously described, substantially greater effort was expended in the cloning and transformation of large constructs (**Chapter 5**: OX5195/OX5241/OX5242/OX5257). This suggests that the system could be improved by using CRISPR to remove the inverted terminal repeats from standard, two-ended *piggyBac* vectors. However, it would be possible to achieve the same results, by adapting the current 4-PB system to a marker-free state. In at least one line, the efficiency of the desired *piggyBac* end excision (loss of the two *piggyBac* paired ends, with retention of the central element) was about 6% in *P. gossypiella* (Morrison, 2007). Though this is expected to vary with different genomic insertion sites and insect species, it does demonstrate that a marker-free 4-PB system is theoretically, a viable alternative in Medfly (Dafa'alla et al., 2006), (Condon et al., 2007). It is interesting to note that the 4-PB system removes all *piggyBac* sequence, at both pairs of ends. There would be mechanistic difficulties associated with the full deletion of both ends from a two-ended construct with CRISPR. First, it would be necessary to apply three sgRNAs. The first would be targeted to the inverted terminal repeat (ITR) present at both ends. The second and third would, respectively, target sequences within the central element of the construct that border the *piggyBac* 5' and 3' elements. This would incur a risk of deleting the whole construct; CRISPR can mediate deletions of more than 200 kb (Zhou et al., 2014). This risk is impossible to quantify without further experimentation. Alternatively, deletions may be associated with inversions (Kraft et al., 2015). This could theoretically be problematic, because the expression of tetracycline-repressible transgenic systems is affected by its position relative to cis-acting genomic elements (Schönig et al., 2011). It is interesting to note that the removal of one ITR is expected to completely block transposition (Li et al., 2001a), and that the results of the fluorescent marker mutagenesis experiments demonstrated that this would be possible with acceptable efficiency. However, it is commercially difficult to justify the application of products that retain more piggyBac sequence than previous constructs, from which all pB sequence was removed with the 4-PB system. Therefore, the more simple option would be to first test the viability of a marker-free 4-PB system.

In terms of generating piggyBac sequence-free insertions, equivalent results to the 4-PB system or CRISPR-mediated deletion, could be achieved with site-specific integration. There are additional benefits: complete independence from *piggyBac*

vectors, and potentially improving the efficiency of product development by localising effectors to sites where the transgenic expression profile is already known. This study has characterised a small library of genomic insertion sites that confer a favourable expression profile to transgenes in the male germline (OX5122, OX5123, OX5195 and others; **Chapters 3-5**) and one for the female germline (OX5173D). Though the genomic insertion site is not yet known for many, it would be simple to isolate them; the efficiency at which novel expression systems could be integrated to these sites, however, remains to be demonstrated. Site-specific integration of a 1.2 kb fragment was observed in 11% of progeny for a particularly effective combination of sgRNAs and homologous template DNA in *D. melanogaster* (Gratz et al., 2014). Into mammalian cell culture, targeted integration of a 4.5 kb fragment was achieved (Chu et al., 2015). We are not aware of any reports in fruit flies achieving integration of fragments of equivalent size, but it remains theoretically possible. The efficiency of site-specific CRISPR integration remains an important consideration, because a commercially applicable male sterility phenotype is generally isolated from 5-10 random *piggyBac* insertions, for a functional expression construct. Therefore, the probability that this strategy will be commercially applicable, requires further experimentation to determine this efficiency.

The present dispute for patent rights of the CRISPR platform (Ledford, 2016) casts uncertainty about the extent to which these systems can be applied to commodities protected as intellectual property in future (such as products developed at Oxitec). Potentially, licencing agreements could be necessary even if CRISPR is not a functional element of the final product (for instance, if they were only used for site-specific integration, or the removal of piggyBac sequences). It is furthermore unclear whether the application of CRISPR/Cas9 variants with altered specificity or function will be subject to the restrictions that may be applied to the original *S. pyogenes* CRISPR system. Regardless, it is clear that the ability to easily modify or functionally adapt targeted sequences of DNA can be powerfully applied to study genetic function, develop bioproducts and potentially address debilitating diseases. If implemented responsibly, the technology will undoubtedly have profound positive effects on society.

Chapter 7 – Discussion

7.1 Summary of the study and its objectives

We intended to develop transgenic systems for a population control strategy for Medfly similar to the sterile insect technique (SIT), but addressing its known limitations (**Section 1.4**), by the targeted expression of transgenes to mediate sterility and improve the ability to monitor strain performance in the field. The SIT has been applied with varying degrees of success against many invasive pest species (**Section 1.3.7**), including Medfly, across the world (Vanderplank, 1947), (Lindquist et al., 1992), (Vreysen et al., 2000), (Kohama et al., 2003), (Oliva et al., 2012). The primary requirement of a successful programme is the reliable induction of male sterility and the release of enough sterile males to reliably overwhelm the capacity of wild females to accept fertile, wild males (Knipling, 1955), (Parker and Mehta, 2007), (Black et al., 2011). The current practice of irradiation reliably achieves 99-100% male sterility (Robinson et al., 2002), (Bakri et al., 2005), (Mastrangelo et al., 2010). However, it is not specifically directed to the male germline. Therefore, it damages non-target tissue and undesirably reduces parameters such as lifespan and mating competitiveness, which must be retained to an acceptable level for sterile males to mate with wild females, at an acceptable frequency (Hafez and Shoukry, 1972), (Shelly et al., 1994), (Lance et al., 2000), (Alphey, 2006).

These undesirable effects on male competitiveness vary between insect species; for instance, moths are very tolerant to irradiation (Hallman, 2004). The conditions of irradiation, such as dosage and the developmental stage of the insect, also affect the extent to which competitiveness is reduced (Anwar et al., 1971), (Fisher, 1997), (Robinson et al., 2002), (Alphey, 2006), (Hallman et al., 2010). Furthermore, competitive ability may be adversely affected by the genetic and behavioural effects of colonisation and adaptation to the lab, and the stressful conditions of mass rearing (Bush et al., 1976), (Ozaki and Kobayashi, 1981), (Liimatainen et al., 1997), (Briceño and Eberhard, 1998). Mass-rearing is a necessary requirement of this strategy, and therefore its effect must be considered, if a competitive male sterile strain is to be developed. The competitive profile of the released males can be improved, for instance by exposing males to aromatic compounds prior to dispersal, or periodically renewing the mass-rearing colony with a small population maintained under circumstances more similar to the field (Shelly et al., 2004), (Shelly, 2012). Therefore, the application of an

optimised dose of radiation under appropriate circumstances can reduce side-effects on longevity and mating competitiveness, though it is generally agreed that mass-rearing and sterilisation incur undesirable effects, even in carefully managed scenarios (Hooper, 1972), (Shelly et al., 1994), (Shelly and Whittier, 1996), (Alphey, 2006). This is problematic, as it reduces the efficiency of the strategy and increases the required scale of release. To address these undesirable side-effects, we sought to develop a more targeted method to sterilise males, by the expression of a chimeric nuclease effector (protamine-FokI) in the male germline (Jin, 2011), (Bilski, 2012), (Alphey, 2015). In Chapter 5, two successful transgenic expression systems for repressible male sterility were obtained (OX5242: Ccprot1-short 5'UTR-FokI; OX5257: Dmprot2-chimeric-new-FokI). Both demonstrated a highly penetrant and mostly repressible male sterility phenotype. Furthermore, males of one strain (OX5242(1)H) were confirmed to transfer acceptable quantities of fluorescently labeled sperm to WT females upon mating (**Figure 5.12**), indicating that they were suitable for field use.

To enhance monitoring in the field, it was also desirable to engineer two expression systems for fluorescent marking, one expressed in the body of the insect, and the second specifically in sperm nuclei (Scolari et al., 2008), (Harris et al., 2012), (Asadi, 2013). Several methods for marking the body of insects have been described, for instance with fluorescent powders or the ingestion of dyed food (Wilkinson et al., 1972), (Nestel et al., 2007). The accuracy of these monitoring strategies can be reduced if not carefully managed; the markers can dissipate, reduce male fitness, or be transferred to wild males (Hagler and Jackson, 2001). A highly functional transgenic expression system for bright, whole-body fluorescent marker expression was previously developed at Oxitec: the dsRed2 fluorescent protein is expressed specifically in muscle with components of the Mexfly muscle actin gene (Koukidou et al., 2016). This facilitates the unambiguous differentiation of wild and transgenic males, without apparent effects on male fitness, the risk of dissipation in the field, or the possibility of transferring marking elements to wild males.

A system for fluorescent marking of sperm nuclei would facilitate a direct estimation of mating competitiveness, by scoring the presence of fluorescently marked sperm in females that had mated the transgenic strain (Scolari et al., 2008), (Scolari et al., 2014). It would also allow the identification of the mating partner of wild females: wild males, transgenic males, or both (Scolari et al., 2014), (Chapter 4). Practically, this

is an improvement on the current field practices: adhesive chemical or fluorescent powder can be applied, which are transferred from males to females during copulation (Jarvis and Rutledge, 1992), (Armsworth et al., 2008). These markers can dissipate in the field (Hagler and Jackson, 2001), and importantly, they would not indicate the number of sperm transferred or even that copulation had occurred. Alternatively, the presence of irradiated sperm in the spermathecae can be used to score mating events with sterile males. However, this relies on detectable morphological differences being induced by irradiation, which are known for Medfly (McInnis, 1993), but not all species (Helinski and Knols, 2009). Scoring the head size of sperm is, however, difficult in comparison with assessing the presence of an abundant fluorescent marker. We successfully developed a protamine-based expression system (Ccprot1-zsGreen) that fully labeled mature sperm (**Figure 4.3**), and this was successfully expressed alongside the effector of repressible male sterility and the whole-body fluorescent marker (**Figure 5.12**). Therefore, we were able to generate a single strain with repressible male sterility and fluorescent labeling of the body and sperm, suitable for use in an area-wide control programme for Medfly.

However, it is still necessary to integrate these components with a previously tested and highly efficient genetic sexing strain (OX3864), to engineer automated female removal for a male-only release. Thereafter, males of a transgenic strain expressing all four components (male sterility, fluorescent body marking, fluorescent sperm marking and genetic sexing) must be assessed for their ability to reduce populations of Medfly after sustained mass releases, in field-like conditions (Leftwich et al., 2014). We also described a series of CRISPR-based tools for the precise and highly efficient genetic engineering of Medfly, which could be applied to improve the efficiency of product development, modify previously evaluated transgenic strains for novel characteristics, or study novel sequences of developmental or commercial significance. A discussion of these systems and their practical relevance was previously provided, in Chapter 6 (**Section 6.4**).

7.2 Transgenic expression systems for the fluorescent labelling of sperm nuclei can be applied to assess the mating performance of male sterile strains in the field

7.2.1 Fluorescent marker expression systems regulated by Ccβ2tubulin or Ccβ2tubulin-tTAV do not effectively label sperm nuclei

Three systems regulated by Ccβ2tubulin or Ccβ2tubulin-tTAV (OX3671, OX4282 & OX5036), were not effective in labeling the nuclei of sperm, and hence were not suitable for field use (Jin, 2011), (**Chapter 3**). However, the results were valuable in enabling us to subsequently develop a protamine-based expression system that demonstrated field-applicable properties (**Chapter 4**). The OX3671 fragment was based on a full-length Ccβ2tubulin promoter and 5'UTR fragment (Ccβ2tubulin promoter-Ccβ2tubulin 5'UTR-**dsRed2**-SV40 3'UTR); there was no element providing tetracycline-repressible transcriptional activation (tTAV) in this design. The dsRed2 reporter was visibly detectable in fewer than half of sperm (**Figure 3.1**), (Jin, 2011). This was a curious result, as the Ccβ2tubulin promoter and 5'UTR were shown to mediate bright fluorescent marking in the spermatids and sperm of *C. capitata* by another group (Scolari et al., 2008). Interestingly, the fluorescence observed within the testes of live, undissected OX3671 males appeared comparable to the strains assessed in the Scolari study (Jin, 2011). Therefore, the reasons why we did not observe fluorescent labeling of all sperm were unclear. We demonstrated that the levels of transcription and translation in the male germline, mediated by a modified Ccβ2tubulin fragment (truncated Ccβ2tubulin 5'UTR, fused to Cchsp83 minipromoter-5'UTR), were extremely sensitive to the insertion site of the transposon (OX5036, **Figure 3.11**). Therefore, it was possible that a strain with ability to fluorescently label sperm nuclei equivalent to that described in the Scolari study could be obtained, if a larger panel of lines were assessed. However, this result indicated that stronger and earlier expression of the fluorescent protein would be necessary, to mediate labeling of all mature sperm in the majority of strains tested.

To achieve this expression profile, the Ccβ2tubulin 5'UTR was truncated to remove putative translational delay elements, and attached to a minimal promoter fragment and 5'UTR from Cchsp83 (to mediate earlier transcription and translation). In OX4282, this fragment was used to regulate the expression of tTAV, to transactivate expression of the reporter at the tetO target sequences (tetO14-Dmhsp70 minipromoter-*adh intron*-**tGFP**-SV40 3'UTR). tTAV was adjacent to the tetO operator (the two transcriptional units were cloned in a head-to-head orientation). Therefore, it was considered that the conformation might provide a tetracycline-repressible, positive feedback expression loop, wherein tTAV would activate both its own transcription and that of the tGFP marker, at the tetO target sites. This did not increase the proportion of sperm that were detectably marked (**Figure 3.1**), (Jin, 2011). Therefore, it appeared

likely that a stronger regulatory element, potentially with a translational profile specific to spermatids, would be required to facilitate strong labeling of all sperm nuclei.

We next considered if the application of a fluorescent protein that did not require dimerisation, might be sufficient to enhance labeling of sperm nuclei. It was reasoned that fluorescent proteins requiring dimerisation would demonstrate a non-linear relationship between concentration and activity; in contrast, the activity of a monomeric fluorescent protein would be relatively proportional to its concentration. Therefore, it was possible that the monomeric zsGreen protein might improve the labeling of sperm. In OX5036, tTAV and an nls-zsGreen-nls fluorescent marker were translated as two polypeptides from the same mRNA, separated at the T2A translational skipping element (tetO21-Ccβ2tubulin promoter-5'UTR[short]-Cchsp83 minipromoter-5'UTR-tTAV-**T2A-nls-zsGreen-nls-**Cchsp83 3'UTR). As for OX4282, this conformation was expected to potentially provide a tetracycline-repressible, positive feedback expression loop, wherein tTAV would activate the transcription of itself (tTAV-T2A-nls-zsGreen-nls). Marker expression was extremely weak, and generally absent from sperm. We were therefore surprised to find that this expression system was inferior to the previous systems, which applied tetrameric (dsRed2, OX3647) or dimeric (turbo GFP, OX4282) proteins. It is possible that, despite a non-linear response between concentration and activity, that the aggregation of fluorescent molecules by multimerisation enhances detectability. It is notable that none of these three systems (OX3647, OX4282 and OX5036) applied a fluorescent protein fused to a DNA-binding element. This would be expected to localise the fluorescent molecules to nuclear DNA, and hence improve the visibility. For this reason, it was investigated if protamine-fluorescent marker-FokI fusions could simultaneously localise the fluorescent marker and the male sterility effector to sperm DNA.

7.2.2 Fusions of the male sterility effector and a fluorescent protein are problematic because the components affect the localisation of one another

Two expression constructs (OX4705 & OX4801), were previously tested in olive fly (Asadi, 2013). A linker-free fusion of tetO21-Dmprot2-mCherry-FokI (OX4705) was highly penetrant and repressible (2/4 lines), but marking of sperm nuclei was not observed in any line. Short flexible peptide linkers (SG4) were included between the fluorescent protein and protamine-FokI effector, to introduce a spacer element that might prevent the components from interfering with one another. OX4801

(tetO21-Dmprot2-**SG4 linker**-mCherry-**SG4 linker**-FokI) demonstrated weak but visible sperm marking in all six lines evaluated (**Figure 3.3**). However, the male sterility phenotype was not adequately penetrant (mean hatch rate: 20%). This indicated that both mCherry and FokI were highly sensitive to the conformation of the fusion, as the removal or addition of linker sequences was sufficient to alter the sperm marking and male sterility phenotypes observed.

When the linker-free tetO21-Dmprot2-mCherry-FokI was tested in Medfly (OX4718), we were surprised to find that the results were not equivalent. Penetrant and repressible sterility was observed in 1/2 lines (Bilski, 2012), as expected; however, the fluorescent protein was weakly visible in a minority of sperm, but a majority of late elongating spermatids, in the penetrant line (OX4718A, **Figure 3.5**). This contrasted with the phenotype in olive fly, where fluorescent labeling of sperm was not observed. Therefore, two explanations are possible. The expression systems may behave differently in Medfly and olive fly; this was indicated by a greater intensity of fluorescence in the male germline of Medfly (OX4718), relative to olive fly (OX4801). Alternatively, it was possible that linker-free Dmprot2-mCherry-FokI systems could theoretically facilitate penetrant male sterility and partial fluorescent marking of sperm in olive fly for certain insertions, but it was not observed experimentally, in those lines obtained.

It remained possible that an alternative fusion would be functional. The six possible linker-free fusions, including the one tested (underlined), are: (1) **mCherry**-Dmprot2-FokI; (2) **mCherry**-FokI-Dmprot2; (3) Dmprot2-**mCherry**-FokI; (4) FokI-**mCherry**-Dmprot2; (5) Dmprot2-FokI-**mCherry**; and (6) FokI-Dmprot2-**mCherry**. These combinations could be modified to include an SG4 linker preceding mCherry, an SG4 linker following mCherry, or two SG4 linkers between mCherry and the adjacent molecules. For each scenario, the number of possible combinations increases by six; there are 24 total possibilities. Here, we consider only the linker-free combinations. Prior results indicated that the fluorescence of mCherry was adversely affected by placement between two proteins (Dmprot2-mCherry-FokI), particularly if flexible linkers were not present between mCherry and the two proteins. Therefore, it appeared likely that the alternative fusion with mCherry in the middle of the three proteins (FokI-mCherry-Dmprot2, instead of Dmprot2-mCherry-FokI), would also function poorly; though this would need to be confirmed experimentally. Similarly, we reasoned that it

was probable that any fusion where FokI was orientated in the middle of the three proteins (Dmprot2-FokI-mCherry or mCherry-FokI-Dmprot2), might not mediate substantial endonuclease activity. This is because dimerisation between the FokI cleavage domains is required for DNA endonuclease activity (Vanamee et al., 2001). Therefore, placing FokI in the middle of the fusion protein could potentially prevent dimerisation, by increasing the relative distance between the FokI monomers. This may be an oversimplified explanation, because the chromatin in sperm is highly compacted and spatially aggregated (Kost et al., 2015). Therefore, it remains theoretically possible that fusions with FokI in the middle would still be able to dimerise and therefore cleave DNA.

It is interesting to note, that in a variation of the CRISPR system, N-or C-terminal fusions of FokI to defective Cas9 nuclease (dCas9-FokI and FokI-dCas9) were both able to mediate relatively efficient targeted endonuclease activity (Guilinger et al., 2014b), suggesting that FokI-Dmprot2-mCherry or mCherry-Dmprot2-FokI could potentially be functional. Interestingly, an mCherry-lac repressor-FokI fusion mediated DNA binding, endonuclease activity and visible fluorescent marker expression (Shanbhag and Greenberg, 2013), which would be the features required of our effector molecule. This strongly suggested that a functional mCherry-Dmprot2-FokI fusion could be developed. These were not developed, however, because we reasoned that it would be simpler to design protamine-fluorescent protein fusions (regulated by protamine promoter, 5'UTR and 3'UTR) in a separate module from the effector protein. The functionality of these protamine-based fluorescent protein expression systems was subsequently confirmed (Chapter 4).

7.2.3 Protamine-fluorescent marker fusions regulated by Ccprot1 or Ccprot2 (OX5122, OX5123 and OX5150) visibly localise to the nuclei of all sperm

The translational profile of the *D. melanogaster* protamine-like genes was known to be strong and specific to elongating spermatids, with persistence of the translated protein to mature spermatozoa (Jayaramaiah Raja and Renkawitz-Pohl, 2005), (Barckmann et al., 2013). Therefore, we reasoned that placing a fluorescent protein under the transcriptional and translational control of *D. melanogaster* or Medfly protamine-like genes (promoter-5'UTR-fluorescent protein-3'UTR) would provide strong and constitutive fluorescent sperm marking. A constitutive (non-repressible) fluorescent sperm marking system would enable a more powerful assessment of the

effects of a protamine-FokI effector, in the induced (off-tet) and repressed (on-tet) states, when both components were expressed from the same piggyBac vector. This was because fluorescently marked sperm are more easily recognised than unmarked sperm, and hence it would be easier to detect an effect of protamine-FokI expression on the quantity and morphology of sperm transferred to females, after mating. Because there is an inverse relationship between the quantity of sperm transferred and female desire to re-mate (Mossinson and Yuval, 2003), this would provide useful data regarding the suitability of this two-component transgenic system (repressible male sterility and fluorescent sperm marking) as a measure for population control. We assessed the suitability of three protamine-like genes to localise a fluorescent marker (zsGreen or mCherry) to spermatids and mature sperm: Dmprot1, Ccprot1 and Ccprot2.

None of four lines of the Dmprot1-zsGreen expression system (OX5140) had visible zsGreen fluorescence in testes, despite the demonstration of Dmprot1-zsGreen transcription in one line (OX5140E). The 3'UTR was unlikely to be responsible, because a minimal 3'UTR paired with the Dmprot1 promoter and 5'UTR is sufficient to mediate translation in elongating spermatids of *D. melanogaster* (Jayaramaiah Raja and Renkawitz-Pohl, 2005), (White-Cooper, 2009). The Dmprot1 coding domain is known to mediate DNA-binding in *D. melanogaster* (Jayaramaiah Raja, 2005), (Jayaramaiah Raja and Renkawitz-Pohl, 2005); and the virtually identical Dmprot2 protein was confirmed to bind DNA in Medfly (OX4718, Dmprot2-mCherry-FokI, **Figure 3.5**). However, it is interesting to note that this fusion of mCherry to the C-terminus of Dmprot2 was poorly tolerated (weak fluorescence). Therefore the C-terminal fusion of Dmprot1 to zsGreen may have similarly been responsible for the lack of observed fluorescence. It was additionally possible that the Dmprot1 5'UTR is not functional in Medfly (this was never experimentally confirmed). Notably, we did not observe a translational delay mediated by Dmprot2 5'UTR (OX4718; **Figure 3.5**), though in this instance, it was possible that the adjacent Dmhsp70 5'UTR blocked the ability of Dmprot2 5'UTR to mediate translational delay. The mode by which Medfly protamines are translationally repressed is not known; therefore it is not possible to conclude if the Dmprot 5'UTRs would be functional in Medfly, without further analysis. Because the Ccprot1 and Ccprot2 5'UTR fragments were subsequently indicated to mediate translational repression (**Figure 4.3-4.4**), extensive troubleshooting of the Dmprot1-zsGreen expression system was not a valuable expenditure of resources.

Ccprot2-zsGreen (OX5150) was functional as a fluorescent sperm marking system in one line of three lines. This did not appear to result from an inherently poor performance of the construct. Both lines that did not demonstrate localisation of zsGreen to the male germline were y-linked insertions, which generally perform poorly in the male germline. For instance, transgenic effectors were poorly expressed in the male germline of other y-linked transgenic lines (OX5036AG, OX5195(2)BA1 and OX5257G1: **Figure 3.9**, **Figure 5.3**, **Figure 5.14**). Nonetheless, the results demonstrated that the promoter, 5'UTR and 3'UTR of Ccprot2 were able to mediate localisation of the zsGreen reporter to the elongated spermatid stage, in lines with a favourable genomic insertion site (the phenotype was equivalent to that observed for Ccprot1). This was important, because it indicated that the Ccprot1 and Ccprot2 5'UTR elements would be sufficient to engineer a translational delay of a protamine-FokI effector, and therefore reduce the likelihood of adverse effects on the quantity and morphology of sperm transferred to females after mating. This was subsequently investigated, and a suitable effector derived for Ccprot1-FokI, but not Ccprot2-FokI (**Figures 5.10-5.11**).

The Ccprot1-zsGreen (OX5122) system mediated strong localisation of zsGreen to all sperm nuclei, in every line tested. We were surprised to find that the localisation of Ccprot1-mCherry (OX5123) appeared to be more sensitive to insertion site than that of Ccprot1-zsGreen (OX5122); the mCherry reporter was visibly localised to sperm nuclei in only 3/5 lines. The only differences between these two constructs were the fluorescent protein applied (mCherry/zsGreen); the alteration of two amino acids in the linker peptide between Ccprot1 and the fluorescent protein (OX5122: RYRST; OX5123: RYRY); and the absence of the N-terminal methionine from mCherry in OX5123. Both proteins were monomeric, and hence expected to demonstrate comparable intensity of fluorescence, if expression levels were similar. The expression of Ccprot1-mCherry in a given strain was binary (either highly visible, or not visible at all). Therefore, the observed lack of fluorescence in certain OX5123 lines seemed to be entirely a consequence of the insertion site, rather than a relative weakening of expression caused by alteration of the linker, removal of the N-terminal methionine of mCherry, or the possibility that mCherry was inherently less bright than zsGreen. We observed that certain autosomal insertion sites were superior to others, for mediating high levels of expression in the male germline of Medfly (OX5036: **Figure 3.9**). Because a small number of lines were assessed from OX5122 and OX5123 (five each),

it is possible that two weak autosomal insertions were derived by chance for OX5123, and that similarly weak autosomal insertions might have been observed for OX5122, had a larger panel of lines been screened. It would be possible to directly test this hypothesis, by characterizing the genomic insertion site of a weak OX5123 line (eg. OX5123G), using CRISPR-mediated site-specific integration to direct the OX5122 construct to this locus, and observing the subsequent localisation of zsGreen in the male germline. However, such an assessment is of limited commercial relevance and would be extremely time-consuming; therefore it was not pursued.

7.2.4 Practical uses of fluorescent sperm marking systems to assess mating competitiveness and reproductive biology

The development of two strains with differentially marked fluorescent sperm (Ccprot1-zsGreen [OX5122] and Ccprot1-mCherry [OX5123]) facilitates powerful analyses of the ability of transgenic strains to engage in wild-type mating behaviour, and thereafter induce refractoriness to re-mating in females; both factors would directly impact the success of this population control strategy in the field. Although a final commercial product would require only one fluorescent sperm marking system, it is useful to possess strains with different fluorescent markers, as this allows the interaction of sperm from multiple partners within the same female to be studied. The practical applications of these systems, and the extent to which they were investigated in this study, are next considered.

Heterozygous and homozygous males of one line (OX5122M [Ccprot1-zsGreen]) were tested for mating competitiveness relative to WT males. No significant difference was observed, indicating that expression of the Ccprot1-zsGreen transgenic system did not severely impact male competitiveness. However, it is acknowledged that the competitiveness of sterile males under laboratory conditions does not accurately reflect performance in the field; mass-reared males appear to demonstrate variations in mating behaviour which may compromise reproductive success, and wild females appear to be more selective than lab-reared females (Liimatainen et al., 1997), (Briceño and Eberhard, 1998), (Lance et al., 2000). Therefore, the suitably of these strains for field use must be fully validated by an assessment under conditions more similar to the field, in large greenhouses with foliage to provide lekking sites (Morrison et al., 2009). Ideally, this analysis would be performed with wild-caught males rather than wildtype. The use of wildtype males as competitors is expected to overestimate the

competitiveness of the sterile line, because the wildtype strain is likely to demonstrate lab adapted behaviour and equivalent reductions in fitness associated with lab colonisation and rearing, which may still disadvantage these males in the field. Because the inclusion of a male sterility effector was previously shown to affect the motility and quantity of sperm transferred (OX4718: **Figure 3.8**), there was limited value to performing an intensive assessment of mating competitiveness, before a transgenic strain expressing both components (the male sterility effector and fluorescent marking system) was generated. Two strains (OX5242(1)H1 and OX5257V1) were generated at the end of the study, but time was insufficient to assess them.

The quantity and morphology of sperm transferred to females after mating did not appear to differ between wild-type males, heterozygous transgenic males (OX5122D, G, K & M) or homozygous transgenic males (OX5122M). This indicated that expression of the transgenic marker system did not visibly affect the ability of sperm to behave normally in the female reproductive tract. Furthermore, the ability of males of several transgenic lines to consistently transfer fluorescently marked sperm to females after mating, at similar quantities to WT, indicated that the system would be likely to function reproducibly for most autosomal insertions. A minimally insertion sensitive phenotype was ideal for commercial development, because it reduces the effort required to generate a strain with a commercially applicable phenotype. It should be noted that these modes of scoring were not quantitative, and a strong relationship between the number of sperm transferred to females and their desire to re-mate, has been demonstrated (Mossinson and Yuval, 2003). Therefore, it would be useful to develop a more quantitative mode of scoring; two methods are suggested. First, it would be possible to manually count dilutions of dissected spermathecal tissue under high magnification. Typically, this involves staining sperm with a fluorescent dye such as DAPI, but this could be omitted, as the sperm are already fluorescently marked. Alternatively, qPCR has been applied to quantify sperm DNA; such a method has been developed in the Tephritid pest *A. suspensa* (Fritz et al., 2010). The latter would be more likely to provide an immediate solution, as sufficiently high resolution microscopes are expensive.

In the field, it will be necessary to assess the rate at which transgenic sterile males mate wild females. Therefore, the fluorescent sperm marking system must demonstrate adequate persistence in the reproductive tract of females captured on traps,

and it must be possible to reliably detect the fluorescence after dissection. To evaluate these parameters, females which had been mated to WT or OX5122M-homozygous [Ccprot1-zsGreen] males were left on traps and subsequently dissected. Even after 2 weeks, it was possible to assess whether the female had mated a WT or OX5122M-homozygous male, with 95% accuracy. Across all timepoints (0, 3, 7 and 14 days), the rate of false negatives (mating events to OX5122M males, misidentified as WT because fluorescence was not observed) was considerably higher than the rate of false positives: 5/79 and 2/79, respectively. This was presumably due to the fact that false negative scoring was likely to occur where sperm transfer upon mating was minimal; sperm degradation was high; or sperm were lost during dissection. The only instance in which false positives are likely to occur is if spermathecal autofluorescence is mistaken for fluorescent marker expression. A false positive (1/20) was scored for each of the two early timepoints, but not subsequently; therefore, it appears that this type of erroneous scoring can be reliably eliminated with practice. Therefore, although most events are scored correctly, mis-scoring events are slightly biased towards false negatives. In practical terms, this would lead to a slight underestimation of sterile male competitiveness, because occasionally mating events between transgenic males and wild females would be attributed to wild males. This would be preferable to the alternative scenario (an overestimation of competitiveness), because in practical terms it would be preferable to release sterile males in excess of the minimum required. It should be noted that under a microscope with sufficient resolution, it would be possible to score a complete absence of sperm (rather than an absence of fluorescence), and therefore substantially reduce the rate of false negatives.

Medfly females frequently accept second mates in the wild (Bonizzoni et al., 2002), which would reduce the efficacy of sterile male release for population control, if females frequently re-mated with fertile wild males. This is furthermore problematic, because the majority of offspring are sired from the male mated most recently; about 70-80% for the first five days, though this proportion declines thereafter (Bertin et al., 2010), (Scolari et al., 2014). The likelihood of female re-mating is additionally enhanced if the quantity of sperm transferred is reduced (Mossinson and Yuval, 2003). This is an important consideration because we determined that males sterilised by expression of protamine-FokI transfer, on average, reduced quantities of sperm with lesser motility (OX4718: **Figure 3.8**; OX5195: **Figure 5.5**; OX5242: **Figure 5.12**). Notably, we did not detect such an effect when the fluorescent sperm marking system

was not expressed with an effector of male sterility (OX5122M heterozygotes and homozygotes: **Figure 4.9**). Therefore, it appears that expression of the male sterility effector, but not the fluorescent sperm marking system, has a deleterious effect upon sperm quantity and motility.

Re-mating tendency was investigated in females which were first mated to WT or OX5122M males, and then provided the opportunity to mate a second time, with males of the opposite genotype to which females had first mated (**Section 4.3.2**). We did not observe a substantial difference in re-mating tendency, between the two groups. However, the sample size was small, because most females did not remate (**Table 4.10**). The additional limitations of the study, were that females were not assessed for re-mating over a long period (they were only provided the opportunity to remate once, on the subsequent day). As females are more likely to re-mate after their stores of sperm are relatively depleted (Twig and Yuval, 2005), it is likely that a substantially greater proportion of females would choose to remate one or two weeks later, for instance. Finally, the limitations of assessing competitiveness and re-mating under the artificial conditions of the laboratory, as described in the prior evaluation, apply here. We confirmed that females mated twice (to WT and OX5122) contained sperm of both genotypes, which could be differentiated by the presence or the absence of the fluorescent protein. This indicated that double mating events of this nature could potentially be scored reliably, in the field. However, further analysis with double blind scoring should be performed, to confirm that these events can be accurately scored if the male(s) to which the female has mated, are not known to the experimenter. It was not possible to draw precise conclusions of the ratio of unmarked to unmarked sperm, at the microscope resolutions applied in the study. Therefore, to differentiate the quantities of sperm transferred from first and second male partners, it would be necessary to perform counting at a higher resolution. This would be relatively laborious, and therefore it would be preferable to apply a qPCR-based assay to improve throughput, as previously described.

The last-mate precedence in paternity after multiple mating is well-documented in Medfly, and thought to be mediated by stratification of sperm in the fertilisation chamber (Bertin et al., 2010), (Scolari et al., 2014). After mating, the proportion of sperm in the fertilisation chamber reaches peak concentration, declines over several days, and is replenished by the spermathecae continuously (Twig and Yuval, 2005).

Therefore, after a secondary mating event, the quantity of sperm from the most recent mate is proportionally greater in the fertilisation chamber, through which eggs pass. The last-mate precedence subsequently declines, as the chamber is depleted and replenished by the spermathecae (Scolari et al., 2014). It would have been interesting to assess the dynamics of re-mating in females mated to sterile males, as it would be expected that a sterile transgenic strain able to induce last-mate precedence of paternity, would more efficiently suppress the wild population (a greater proportion of eggs would be fertilised with genetically sterile sperm). For this type of study, it would be extremely useful to apply males with differentially marked fluorescent sperm; two sets of crosses could be performed. In the first set, females would be crossed to a strain expressing a fluorescent sperm marking system (Ccprot1-zsGreen) and a male sterility effector (tetO-protamine-FokI); and subsequently to a second line expressing a different fluorescent marker in sperm (Ccprot1-mCherry), but no male sterility effector. The second set of crosses would be the reciprocal of the first set. Assuming that the fitness penalties associated with expression of each fluorescent sperm marking component (Ccprot1-zsGreen or Ccprot1-mCherry) were equivalent, it would be possible to unambiguously assess the ability of sterile males to induce refractoriness to re-mating in females. In this analysis, the females could be monitored over a series of days to determine the extent to which they accepted secondary mates, and how this was affected by the genotype of the first partner (whether or not the first male was sterile). A particularly powerful analysis would account for other potential factors. For instance, it would be possible to dissect the spermathecae to correlate re-mating tendency with the quantity of sperm transferred upon mating. Furthermore, it would be possible to assess the extent to which last-mate precedence was induced by monitoring the proportion of inheritance of the zsGreen or mCherry transgenes in progeny (the developing eggs could be assessed by PCR to determine whether the father carried either the Ccprot1-zsGreen or Ccprot1-mCherry transgene); and to subsequently correlate this effect with the proportion of Ccprot1-mCherry and Ccprot1-zsGreen marked sperm present in the fertilisation chamber. A similar strategy has been successfully applied in a published study (Scolari et al., 2014). Outside of the context of developing a population control strategy based on sterile male release, the re-mating behaviour of Medfly is complex, scientifically interesting, and not fully characterised despite considerable study (Bonizzoni et al., 2002), (Shelly and Kennelly, 2002), (Kraaijeveld and Chapman, 2004), (Kraaijeveld et al., 2005). Therefore, these transgenic lines may provide an interesting opportunity to address biologically interesting questions.

7.3 Development of a suitable protamine-FokI effector for penetrant and repressible male sterility

7.3.1 The OX4718 male sterility effector (Dmprot2-mCherry-FokI) mediates penetrant and repressible male sterility, but the quantity and quality of sperm transferred to females after mating is reduced

Having described a functional system for fluorescent sperm marking, we sought to combine this with a penetrant and repressible male sterility effector. OX4718 was tested prior to the development of the Ccprot1-zsGreen fluorescent sperm marking system, and was primarily designed from components of the OX4705 construct tested in olive fly (tetO21-Dmprot2-mCherry-FokI), previously shown to mediate penetrant and repressible male sterility (**Figure 3.3**), (Asadi, 2013). The male sterility of OX4718A was highly penetrant (99%) and fully repressible (**Figure 3.5**). The OX4718A insertion was thereafter immobilised by transposition of the *piggyBac* ends ("resolved"), and it was confirmed that the penetrant and repressible sterile phenotype was not affected by the immobilisation (**Figure 3.6**). In lab-scale mating competition assays, WT females did not demonstrate a significant preference for OX4718A-resolved males or WT males. As previously discussed, the mating behaviour of Medfly reared in the lab is not an accurate indication of the wild equivalent; and therefore, further assessment was required to confirm that it could compete well with wild males in field conditions. This was not performed, because we subsequently discovered an issue with the sperm produced by the strain.

We determined that OX4718A-resolved males transferred lesser quantities of sperm in most instances, relative to WT males (**Figure 3.8**). The sperm present in females mated to OX4718A-resolved males frequently demonstrated an unusual, uncurled morphology; were often trapped in the oviduct; and generally immotile. These factors indicated that the sperm were weakened or dead. This was concerning, for two reasons. First, it indicated an increased likelihood of subsequent re-mating in females to which transgenic males had mated, because the quantity of sperm present in the female reproductive tract was reduced (Mossinson and Yuval, 2003). Secondly, it indicated that sterility in the system was not always mediated, or even potentially not primarily, by paternal effect lethality, but rather a lack of sperm transfer, and of fertilisation itself.

The viability of embryos formed by fertilisation with protamine-FokI expressing sperm would be expected to be essentially zero, thereby incurring a severe cost to female reproductive fitness, and increasing the efficacy of the population control strategy. However, if such fertilisation events did not occur or occurred only infrequently, then subsequent re-mating would completely or almost completely reverse the effect of mating to a sterile male. To investigate the proportion of embryos that demonstrate a paternal effect lethal phenotype, a nuclear staining assay was subsequently developed to monitor the extent of development (**Figure 5.7**). It was not applied to this line, because superior lines were simultaneously isolated (OX5242(1)H1 and OX5257V1; **Figure 5.12** and **Figure 5.14**).

The negative effects on sperm transferred to females by transgenic males were partially explained by the pattern of effector localisation (Dmprot2-**mCherry**-FokI). mCherry fluorescence was generally present in late spermatids, but individualised sperm were not visibly marked; this indicated a variability in Dmprot2-mCherry-FokI expression (or its stability) between sperm. This may explain why a minority of sperm are motile, or able to mediate the development of embryos to the larval stage (1% of embryos hatched). Although we noticed that sperm transferred to females that had mated OX4718A-resolved males were frequently unhealthy, such an effect had not been detected during dissection of testes from the OX4718A-resolved line. These were conducted at a relatively early stage of the study, and it is therefore possible that an experienced person would be able to revisit the experiments and detect potential impacts on sperm, when examined in the male germline. This is an important consideration, because the ability to detect defects in the male germline would facilitate the identification of poorly performing strains, at an earlier stage. Finally, we frequently observed visible translation of the reporter in spermatocytes. This was not ideal, because early translation of Dmprot2-FokI would be anticipated to disrupt the meiotic divisions, or cause significant defects in spermatid nuclear shaping, leading to failure of spermatid individualisation.

There were two primary explanations for the presence of translation in spermatocytes, which should not occur if the Dmprot2 5'UTR functions in Medfly as it does in *D. melanogaster* (Jayaramaiah Raja and Renkawitz-Pohl, 2005*)*, (Barckmann et al., 2013). First, it was possible that the Dmprot2 5'UTR is not sufficient to mediate equivalent translational delay in the male germline of Medfly. This was indicated by the

visible translation of the fluorescent reporter in spermatocytes. Dmprot1 or Dmprot2 reporter constructs in *D. melanogaster*, which feature the Dmprot promoter-5'UTR fragment linked to a fluorescent reporter and minimal 3'UTR, do not demonstrate this phenotype (Jayaramaiah Raja and Renkawitz-Pohl, 2005). The mechanism of translational repression of Medfly protamine-like genes is not known; therefore it cannot be guaranteed that the Dmprot2 5'UTR mediates translational delay effectively in Medfly. However, there is a confounding factor: a partial Dmhsp70 5'UTR (89 bp) immediately preceded the Dmprot2 5'UTR. This may have contributed to early translation, as observed when the Ccβ2tubulin 5'UTR was truncated and attached to a Cchsp83 minipromoter-5'UTR fragment in OX4282 (**Figure 3.1**), (Jin, 2011). This could potentially remove the ability of the Dmprot2 5'UTR to mediate translational delay, if the Dmprot2 5'UTR was indeed functional in Medfly. To address these issues, the Dmhsp70 5'UTR was truncated in subsequently evaluated constructs (OX5195/5241/5242). Furthermore, we applied regulatory sequences from Medfly protamines (the 5'UTR, coding domain and 3'UTR of Ccprot1 or Ccprot2) in these constructs, in an attempt to include sequences that might adequately delay the translation of the male sterility effector (protamine-FokI).

7.3.2 Ccprot1-full 5'UTR-FokI (OX5195) mediates penetrant male sterility but is not repressible, presumably due to a minimal promoter fragment within the Ccprot1 5'UTR

The localisation of Ccprot1 promoter-Ccprot1 5'UTR-Ccprot1 ORF-**zsGreen**-Ccprot1 3'UTR (OX5122) indicated that the fragment mediated an appropriate translational delay in the male germline; zsGreen was visible in the nuclei of elongating spermatids and individualised sperm, but not in spermatocytes. When the Ccprot1 promoter was replaced with the full-length Dmhsp70 promoter (OX5184: Dmhsp70 promoter-Ccprot1 5'UTR-Ccprot1 ORF-**zsGreen**-Ccprot1 3'UTR), specific translation in spermatids was still observed. This confirmed that translational repression was conferred by elements within an untranslated region. This was presumably the 5'UTR, because in *D. melanogaster*, sequences within the 5'UTR of Dmprot1 and Dmprot2 are responsible for this translational delay (Jayaramaiah Raja and Renkawitz-Pohl, 2005); this applies to other genes such as *dj* and *Mst87F* (Kempe et al., 1993), (White-Cooper, 2009).

To engineer a translational delay absent in the previously tested strain (OX4718), we therefore developed a Ccprot1-FokI male sterility effector. A tetO21-

Dmhsp70 **mini**promoter-Ccprot1 5'UTR-Ccprot1 ORF-**FokI**-Ccprot1 3'UTR fragment was cloned in a head-to-head configuration with the previously described tetracycline-repressible, male-germline specific expression system (tetO21-β2T-Cchsp83-tTAV). This was performed to facilitate tetracycline repression of the Ccprot1-FokI effector. The resulting construct, and all those tested subsequently, incorporated the Ccprot1-zsGreen sperm marking system as a separate module, to mediate the assessment of male mating competitiveness. The resulting OX5195 expression construct (tetO21-Ccprot1-full 5'UTR-FokI; Ccprot1-zsGreen) demonstrated a highly penetrant male sterility phenotype in certain lines (3/8 were highly penetrant; 2/8 were semi-penetrant). We were surprised to find that the tetO21-Ccprot1-FokI effector was generally insensitive to tetracycline, which did not improve the hatch rate of progeny of OX5195 males; with the exception of two semi-penetrant lines (these were fully repressed).

Several observations suggested that a cryptic promoter element within the Ccprot1 5'UTR fragment was present: prior constructs applying a tetO21-Dmhsp70 minipromoter-Dmprot2-FokI fragment (eg. OX4718/5036/4353) were fully repressible; sensitivity to tetracycline was observed in semi-penetrant lines; multiple transcriptional start sites were indicated by 5' RACE (**Section 5.2.7**); and *D. melanogaster* genes with promoter elements in the 5'UTR are known (Kempe et al., 1993). This hypothesis was strongly supported by the subsequent isolation of repressible strains, where the Ccprot1 5'UTR was truncated (OX5242: Ccprot1-short 5'UTR-FokI). Therefore, it appeared that the tetO-Dmhsp70 minipromoter was fully or substantially repressed, but a secondary promoter acting through a downstream transcriptional start site, was active even in the presence of tetracycline. We reasoned that truncating the 5'UTR fragment could remove or weaken this putative secondary promoter, and therefore engineer a greater extent of repressibility. It would have been possible to characterise the transcriptional start site (TSS) or sites of the Ccprot1-FokI effector, but given the inconclusive results in identifying the TSS of Ccprot1 and Ccprot2 in a prior study (**Figure 4.10**), and the commercial pressures to rapidly engineer a repressible line, we opted to truncate the predicted 5'UTR sequence and attempt removal of these elements.

7.3.3 Shortening the 5'UTR of Ccprot1 (OX5242), but not Ccprot2 (OX5241), engineers penetrant and mostly repressible male sterility

The 5'UTR fragments of Ccprot1 (OX5242: Ccprot1-short 5'UTR-FokI) and Ccprot2 (OX5241: Ccprot2-short 5'UTR-FokI) were truncated, and each applied to regulate a repressible male sterility system, in a conformation that was otherwise

identical to that previously described (tetO21-Dmhsp70 minipromoter-Ccprot 5'UTR [**short**]-Ccprot ORF-FokI-Ccprot 3'UTR). A single line, OX5242(1)H1 was assessed for the tetO21-Ccprot1-short 5'UTR-FokI system. It demonstrated a penetrant (99%) and mostly repressible (81%) phenotype. This was consistent with the presence of a putative secondary promoter, which had been mostly attenuated. The exact mechanism is uncertain. It could for instance, be mediated by the weakening of the promoter element; or an enhancement of its sensitivity to tetracycline repression, by being brought closer to the tetO sequences. It remains possible that a fully repressible phenotype could be generated, by further shortening of the Ccprot1 5'UTR. This was not pursued because the male sterility phenotype appeared to be adequately repressed, and the commercial benefits of modifying the system to full repressibility, were insufficiently compelling to recommend the investment of further time and resources. If the strain is sufficiently repressible to be mass-reared without effects on the fitness of the colony, then a greater extent of repressibility is not highly advantageous commercially.

The male sterility effector incorporating a shortened version of Ccprot2 5'UTR (OX5241) was extremely penetrant in 3/4 lines (> 99%) but completely non-repressible. Therefore, it appears that the 5'UTR fragment was not sufficiently shortened to remove all putative elements driving tetracycline-independent expression of the construct. It would be interesting to perform 5'RACE on this strain to confirm the presence of transcription from an element within the 5'UTR. Alternatively, it would be possible to generate an even shorter version (for instance, retaining 70 nt of the 5'UTR), and investigate the extent of repressibility. However, this was not necessary commercially, because the Ccprot1 short 5'UTR-FokI effector was suitable for commercial development. There was therefore no need to develop a redundant system, or extensively assess the mechanisms that underpinned the failure of this effector to respond to tetracycline.

7.3.4 Expression of Ccprot1-full 5'UTR-FokI (OX5195) in the male germline blocks the embryonic development of progeny, but at a later stage than expected

We investigated the paternal effect of Ccprot1 full 5'UTR-FokI expression in the male germline, by assessing the embryonic development of progeny of OX5195 (Ccprot1-full 5'UTR-FokI) males (reared off-tet and on-tet) with nuclear staining. We assessed embryos at early (0-4 hours) and late (20-24 hours) timepoints; from personal

observation, embryos of the Oxitec WT strain typically hatch between 60-72 hours. We expected that the majority of embryos from crosses of off-tet reared males of a penetrant line (OX5195(2)AU1) would not develop at all, given the minimal hatch rate observed (1%). From the early stage (0-4 hours), results were as expected; no embryos exhibited nuclear divisions. We were surprised to find that most embryos (62%) of the later timepoint (20-24 hours) demonstrated extensive cellular division that proceeded past the blastodermal stage, and included early tissue differentiation. In *D. melanogaster*, mutants where the paternal genome is prevented from mediating embryonic development have been characterised. This is expected to be similar to the phenotypic conditions induced in these embryos, for which the paternal genome has been damaged by protamine-FokI expression. In homozygous *maternal haploid* (*mh*) mutants, the paternal chromatids do not form functional centrosomes and are excluded from subsequent divisions. About 22% reach a late stage of embryonic development (cuticle deposition) from haploid gynogenetic development, though none hatch (Loppin et al., 2001). Mutants of a gene with a similar phenotype, sésame, do not demonstrate decondensation of sperm chromatin after embryonic entry. As with *mh* mutants, the paternal DNA is excluded from subsequent divisions. Although hatching is not observed, the majority (72%) proceed to late embryonic development and form a cuticle; about 7% arrest in early development (Loppin et al., 2000). To compare to the phenotype we observed in OX5195(2)AU1 [NT] embryos, early arrest was more frequent (38%) and the proportion of embryos demonstrating tissue differentiation was similar (62%). This should not be taken to indicate that the mode of arrest is similar, as we did not perform an extensive cytogenetic analysis. However, it does indicate that even severe disruption of the paternal DNA, to an extent sufficient to induce aneuploidy, is not sufficient to block embryonic development in every instance (Loppin et al., 2001).

Therefore, we conclude that the expression of Ccprot1-FokI in spermatids induces severe double-stranded breaks and prevents hatching. However, it does not prevent partial development of embryos, apparently from the maternal haploid genome primarily. Though unexpected, this is not commercially an issue. Hatch rate was suppressed to 1%, and we never observed eclosion of adult progeny. Therefore, there appears to be no risk of vertical transmission of the transgene in the wild. Furthermore, the incidence of crop destruction from larval hatching will be severely reduced in this instance, and would be eliminated, if sterility is completely penetrant. A strain with full

penetrance in the heterozygous state (OX5257: Dmprot2-chimeric-new-FokI, **Figure 5.15**) was subsequently identified. It will be interesting to assess the embryonic arrest phenotypes in progeny of OX5242 (Ccprot2-short 5'UTR-FokI) and OX5257 (Dmprot2-chimeric-new-FokI) males, which demonstrated superior phenotypes for commercial development: highly penetrant male sterility that was mostly repressible.

It should be noted that the assay was developed to a preliminary state, and could be substantially improved. The sample size should be increased to at least 100 embryos per experimental group at each timepoint, as it was evident that the sample size applied was not sufficiently powerful to detect minor effects on viability (no effect on viability was detected for a semi-penetrant line, which demonstrated a reduced hatch rate). Furthermore, the collection cycles were relatively non-synchronised, spanning four hours. This was problematic, as a confounding factor for the early timepoint (0-4 hours) was that a minority of eggs may have appeared undeveloped because they were recently laid, rather than affected by the expression of protamine-FokI. This effect could be abolished by collecting eggs in a 10-30 minute window. Finally, the phenotypic categories selected for the 0-4 hour collection (no divisions; divisions visible) and the 20-24 hour collection (morphologically normal; morphologically aberrant; or no visible development) were adequate to highlight severe developmental aberrations. However, they were not particularly meaningful from a developmental standpoint, and did not accurately account for the intricacies of development that occur in a 24 hour window. Therefore, it would be preferable for the experimenter to learn to accurately stage embryos using a system similar to the 17-stage model widely used in *D. melanogaster* (Campos-Ortega and Hartenstein, 1997). Finally, it would be interesting to introduce a quantitative element to the study to improve statistical analysis, for instance by quantifying the paternal and maternal genomic copy numbers with a molecular assay. Differentially quantifying the number of paternal and maternal genomes present would also provide evidence to confirm or disprove our hypothesis that embryos derived from protamine-FokI expressing fathers develop primarily as haploid gynogenetic embryos, from the maternal genome alone. Similar qPCR strategies have been applied to quantify the number of viral genomes within insect tissue to determine the likelihood of infectiousness (Walker et al., 2011), (Bian et al., 2013). Therefore, it seems reasonable that such an assay could be adapted to these ends.

We noted that the quantity of sperm transferred to WT females after mating, was

reduced in OX4718, OX5195(2)AU1, and OX5242(1)H1 males reared off-tet (**Figure 3.8**, **Figure 5.5**, **Figure 5.12**). That a proportion embryos developed from crosses of OX5195(2)AU1[NT] males, but did not hatch, indicated that sterility was mediated by paternal effect lethality, in at least some instances. However, it was not possible to conclude whether embryos that arrested with zero visible divisions were the result of a very early paternal effect lethal phenotype, or were merely unfertilised. As the transgenic sperm express a protamine-zsGreen fluorescent sperm marker, it may be possible to visualise the paternal haploid genome within the fertilised egg by fluorescence microscopy. The visibility of Ccprot1-zsGreen associated with the paternal genome could be validated by staining embryonic progeny of males of the Ccprot1-zsGreen line (OX5122) or Ccprot1-zsGreen; Ccprot1-short 5'UTR-FokI line (OX5242). This is certainly worthy of investigation, as it would preclude the necessity for highly sensitive PCR techniques, or the development of alternatively marked transgenic strains, for instance expressing a fluorescent protein in the sperm tail (Santel et al., 1997).

7.3.5 Expression of protamine-FokI reduces the motility and quantity of sperm transferred to females after mating

Because a previous protamine-FokI effector (OX4718: Dmprot2-mCherry-FokI) had demonstrated profound effects on the quantity and quality of sperm transferred to females after mating, we next investigated if males of the recently developed systems had similar deficits. We first assessed the OX5195 effector (Ccprot1-full 5'UTR-FokI**),** which was not repressible in highly penetrant lines. Marked sperm were visible in the most penetrant line (OX5195(2)AU1 [99%]), but at an extremely low quantity. Crosses of the second and third-most penetrant lines (OX5195(2)AC2 [97%] and H1 [93%]) demonstrated an intermediate phenotype. There were comparatively more sperm present relative to OX5195(2)AU1, but fewer relative to the very weakly penetrant lines (OX5195(2)AI1 [34%] and BG1 [16%]) and WT. The morphology, however, appeared similar to WT. The rate of sperm transfer from males of the weakly penetrant or impenetrant lines was similar to WT. Therefore, strongly penetrant male sterility was associated with reduced transfer of sperm to mated females, with an approximately linear, inverse relationship between penetrance of male sterility and the quantity of sperm, at least in OX5195.

We performed a similar analysis on OX5242(1)H1 males (tetO21-Ccprot1-short 5'UTR-FokI), which demonstrated a commercially applicable penetrant and repressible

male sterility phenotype. Crosses of off-tet and on-tet reared OX5242(1)H males to WT females demonstrated that the rates of sperm transfer were markedly reduced in OX5242(1)H1[NT] relative to OX5242(1)H1[T] and WT[NT]. Furthermore, there was a reduction in the number of sperm with visible movement, in crosses of OX5242(1)H1[NT] males. In some instances, sperm that appeared to have been crushed by the cover slip were observed; but these were also present in the WT samples. Therefore, at present, we did not detect any effect on the morphology of sperm. However, it would be interesting to perform further investigation at a higher magnification, and with a haemocytometer or similar device that would not risk crushing any cells under investigation; this would allow the unambiguous confirmation of an effect on morphology, or lack thereof. Despite a reduction in the quantity of sperm transferred upon mating, the transfer of detectably marked sperm in every instance, was an improvement on the prior strain characterised (OX4718A-resolved). It is possible that the ability of the Ccprot1 5'UTR to mediate translational delay, was responsible for this improvement. However, the removal of the Dmhsp70 5'UTR (a gene with early translation) from the effector fragment may have been a contributing factor. Because a comparison of tetO21-Ccprot1-FokI effectors with and without the Dmhsp70 5'UTR were not performed, it is not possible to confirm that its removal had any effect.

Consistent with the incomplete repression observed in the egg hatch assay, the number of sperm transferred to females in OX5242H1[T] crosses appeared to be reduced relative to the WT[T] crosses. As previously described, it was difficult to perform relative quantification under the experimental conditions applied (squashes of spermathecae containing hundreds of overlapping live sperm, directly observed at 10x-40x magnification). It would be interesting to apply a PCR-based strategy to quantify sperm by amplification of transgenic or male-specific sequences; such a technique has been developed for *A. suspensa*, a Tephritid pest (Fritz et al., 2010). The effect of protamine-FokI expression was not assessed in OX5241 (Ccprot2-short 5'UTR-FokI), because it did not demonstrate a male sterility phenotype suitable for commercial development (it was not repressible). Furthermore, it was not possible to assess OX5257 (Dmprot2-chimeric-new-FokI) lines, due to insufficient time. However, the assessment of OX5257 lines will be particularly interesting, as a similar effector (OX4718: Dmprot2-chimeric-old-mCherry-FokI) demonstrated deficiencies in sperm transfer and morphology. This will provide further evidence regarding whether the effect was

systematic, or an insertion-specific phenotype in OX4718A-resolved.

These results provided clear evidence that expression of tetO21-Ccprot1-FokI to an extent sufficient to sterilise males, has undesirable effects on the quantity and motility of sperm transferred to females upon mating, despite the inclusion of Ccprot1 5'UTR elements which were previously shown to mediate appropriate translational delay in a study of fluorescent reporter localisation (OX5184, Chapter 4). Though unexpected, this is not entirely surprising, as the zsGreen reporter (Dmhsp70 promoter-Ccprot1-zsGreen) was translated in spermatids which were not yet fully elongated. Therefore, it could be envisioned that even at this relatively late stage, the translation of Ccprot1-FokI was associated with defects in spermatid nuclear shaping, leading to failure of spermatid individualisation, and therefore producing sperm with aberrations. It should also be noted that the zsGreen reporter was constitutively transcribed from Dmhsp70 promoter, rather than from a tetracycline-repressible positive feedback loop. Therefore, the levels of tetO21-Ccprot1 short 5'UTR-FokI translation in spermatids may have been greater than that of Dmhsp70 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1 3'UTR (OX5184). It would have been highly informative to assess if sperm in the male germline are visibly affected, before they are transferred to females (by dissection of the male germline). There was insufficient time to perform this assessment, but it is recommended for future study.

In practical terms, it will be interesting to assess if the reduction in the quantity of sperm transferred affects the utility of this transgenic system, as a novel means of population control. It is expected that females mated to these transgenic strains will be relatively more likely to remate, given that re-mating is correlated with the quantity of sperm transferred (Mossinson and Yuval, 2003). It will be particularly interesting to develop quantitative methods to assess the number of sperm transferred, for instance the high-resolution microscopic assessment or qPCR strategies previously described, to understand this theoretical effect in detail. It remains possible that a strain with full repressibility and minimal or no effects on sperm will be isolated, from the OX5242 and OX5257 lines which have not yet been evaluated (three each). A female-specific flightless mosquito line developed at Oxitec demonstrated non-repressibility in certain lines and full repression in others (Labbé et al., 2012). Therefore, it remains possible that a fully repressible OX5242 line may be identified, despite the observation that the

repressibility of highly penetrant male sterile lines of a similar construct (OX5195: tetO21-Ccprot1 full 5'UTR-FokI) was similar (all were equally insensitive to tetracycline). However, the possibility of isolating a highly penetrant OX5242 or OX5257 line with minimal or no negative effects on sperm appears low, because expression of tetO-Ccprot1-FokI (OX5195 and OX5242) at levels sufficient to mediate > 90% penetrance, has been associated with negative effects in four lines. Experimentally, it would first be preferable to quantify the extent to which sperm transfer is reduced, and thereafter to describe how this translates into a practical effect relevant to the population control strategy. For instance, if males remain relatively competitive for mating events in the field, and females re-mate only to an extent that can be attenuated by release of sterile males at a greater ratio, then there is minimal commercial benefit to developing a strain with a superior ability to transfer large quantities of sperm. Should the development of such a strain become necessary, it is possible that translation could be further delayed by the incorporation of a 5'UTR conferring a translational delay at a later stage. However, considering the difficulties associated with engineering translational delay with the Ccprot1 5'UTR (non-repressible sterility resulted in the first instance, and took several months to fix), such experiments should only be undertaken if a commercial benefit can be directly confirmed.

7.4 Suggestions for future commercial development

It is now necessary to generate a double homozygous strain, for the male sterility effector and fluorescent sperm marking system (OX5242 or OX5257), and the tetracycline-repressible male-selecting system (OX3864). Thereafter, it must be confirmed that the male sterility and male-selecting (genetic sexing) phenotypes are fully penetrant and sufficiently repressible, when expressed together in a single strain. It would also be useful to investigate the expression patterns of the molecular components of the male sterility effector and male-selecting elements, to determine if the effectors are expressed on-tetracycline or in non-target tissue. This is an important consideration, as tetO-protamine-FokI expressing transgenic lines have previously demonstrated expression on-tetracycline and in somatic tissue (Jin, 2011), (Asadi, 2013), (Megas et al., unpublished data). The leakiness of the tet system is frequently affected by positional effect, and therefore it may be necessary to characterise a variety of insertions to find a suitable candidate for penetrant and repressible expression of both systems with minimal leakiness (Schönig et al., 2011).

The mating competitiveness of the double homozygous strain should first be tested in the laboratory, as previously described, to see if there are any evident defects in mating competitiveness. Finally, a life history analysis of the double homozygous strain will be performed and we will assess the mating competitiveness of the double homozygous strain under field-like conditions (greenhouses), to practically validate the ability of the strain to reduce populations of Medfly. Greenhouse population trials have been conducted for the male-selecting OX3864A-homozygous strain in Austria, Greece, Brazil, Morocco, Australia, and the UK; and an open release permit granted in Brazil (Leftwich et al., 2014), (Oxitec, 2016). Therefore, following the approval of a licence for the novel strain described herein (integrating fluorescent sperm marking, repressible male sterility and repressible male-selection), such a strain could be implemented alongside the current programmes assessing the efficacy of the OX3864A strain. As previously described, this strain is essentially an improvement of the OX3864A male-selecting system, incorporating two additional effectors for repressible male sterility and fluorescent sperm marking. The practical benefits mediated by these additional two features is considerable. First, the larval hatch rate is reduced to 0-1%; progeny of the OX3864A strain hatch at a similar rate to WT; but female progeny die before pupation (Leftwich et al., 2014). Therefore, the novel system would reduce the extent to which fruit would be damaged by larval development, hence providing commercial benefits. Secondly, the rate of adult eclosion of the most penetrant strain characterised to date (OX5257V1) was zero (n=300). Therefore, at present, the risk of vertical propagation of the transgenes in the wild appears to be zero for highly penetrant strains. It will however, be necessary in future to investigate the rates of larval, pupal and adult development in progeny of transgenic males with a larger sample size ($n > 10000$), to confirm that adults never eclose. In practical terms, this is a sufficient improvement on the OX3864A system, for which half of progeny of homozygous transgenic males and wild females, would be expected to survive to adulthood (all male progeny).

Furthermore, a fluorescent marking system in sperm provides a direct method to trace the mating competitiveness of sterile males. This would provide evidence that sterile males were effective in the field, and from an economic and practical perspective, allow the development of models to assess the required scale of release to achieve a desired level of suppression. We anticipate that male-selecting and repressibly male sterile strains with fluorescently marked sperm, demonstrating adequate

competitiveness and acceptance by regulatory bodies, will be an invaluable tool for area-wide population control of Medfly and other insects of economic importance.

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Chapter 8 – Appendix

Appendix 8.1 – Medfly genes homologous to the *D. melanogaster* meiotic arrest genes (tMAC and tTAFs)

Protein sequences of tMAC genes (aly, comr, mip40, tomb, topi, Caf1 [180 kDa subunit], achi, wuc) and tTAF genes (can, mia, rye, nht, sa) from *D. melanogaster* were searched for homology against the Medfly genomic database with an expect threshold of 10 (NCBI, 2016). Matches with a score less than 100 were rejected. The sequences were aligned with M-Coffee (**Figure 8.1**). The *D. melanogaster* homologues were preceded with *Dm* (eg. *Dm-aly*) and the Medfly homologues with *Cc* (eg. *Cc-aly*). A homologous sequence was returned for every gene except *wuc* (tMAC) and *rye* (tTAF). The tTAF like genes may not be correct in every instance; it is possible that the result is the isoform present in the basal version of TAF, rather than the testis-specific version (Hiller et al., 2001). *Cc-can* was annotated as a testis-specific homologue in the NCBI database; *Cc-mia*, *Cc-rye* and *Cc-sa* were not. In an attempt to find the basal and testis-specific versions of these three genes, the Medfly sequences were searched for homology against the Medfly genomic database with a lower stringency (expect threshold: 100). This failed to identify a testis-specific and basal homologue, for all three genes. The Medfly homologues of tMAC and tTAF genes may represent a useful source of regulatory sequences for the targeted localisation of transgenic effectors. Alternatively, they may provide interesting targets for induced male sterility, for instance by RNA interference or synthetic CRISPR repressors. However, it is likely that the sterility phenotypes will not be ideal, because mutants of meiotic arrest genes do not produce mature spermatozoa, therefore females will not be transferred sperm upon mating, and will be likely to remate (Lin et al., 1996), (Mossinson and Yuval, 2003). Nonetheless, the genes may represent a wealth of regulatory sequences for the male germline; alternatively, it would be interesting to generate mutants or fluorescent reporter fusions to characterise their role in male reproductive development.

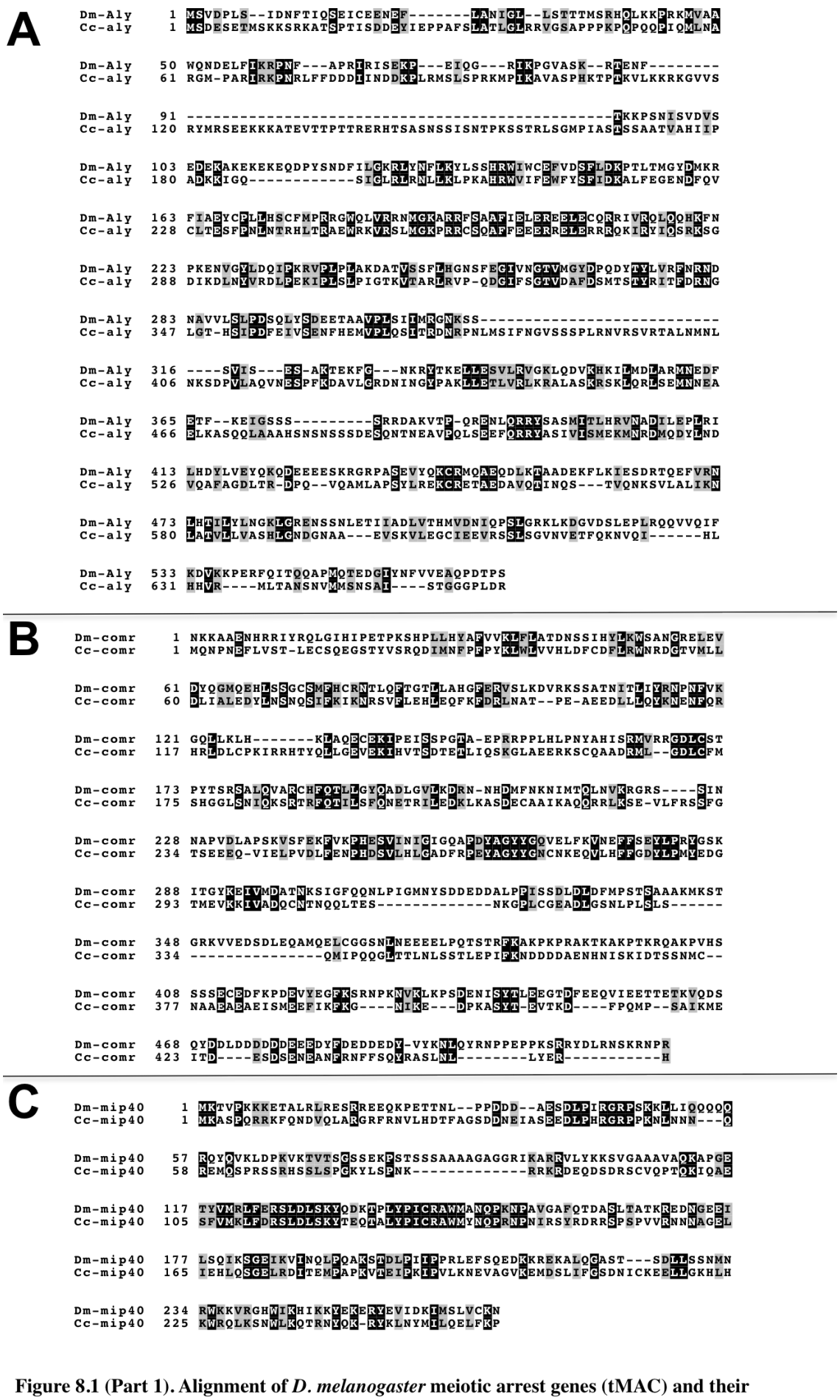

Figure 8.1 (Part 1). Alignment of *D. melanogaster* meiotic arrest genes (tMAC) and their homologues in Medfly. (**A**) *Aly*. (**B**) *Comr*. (**C**) *mip40*.

A

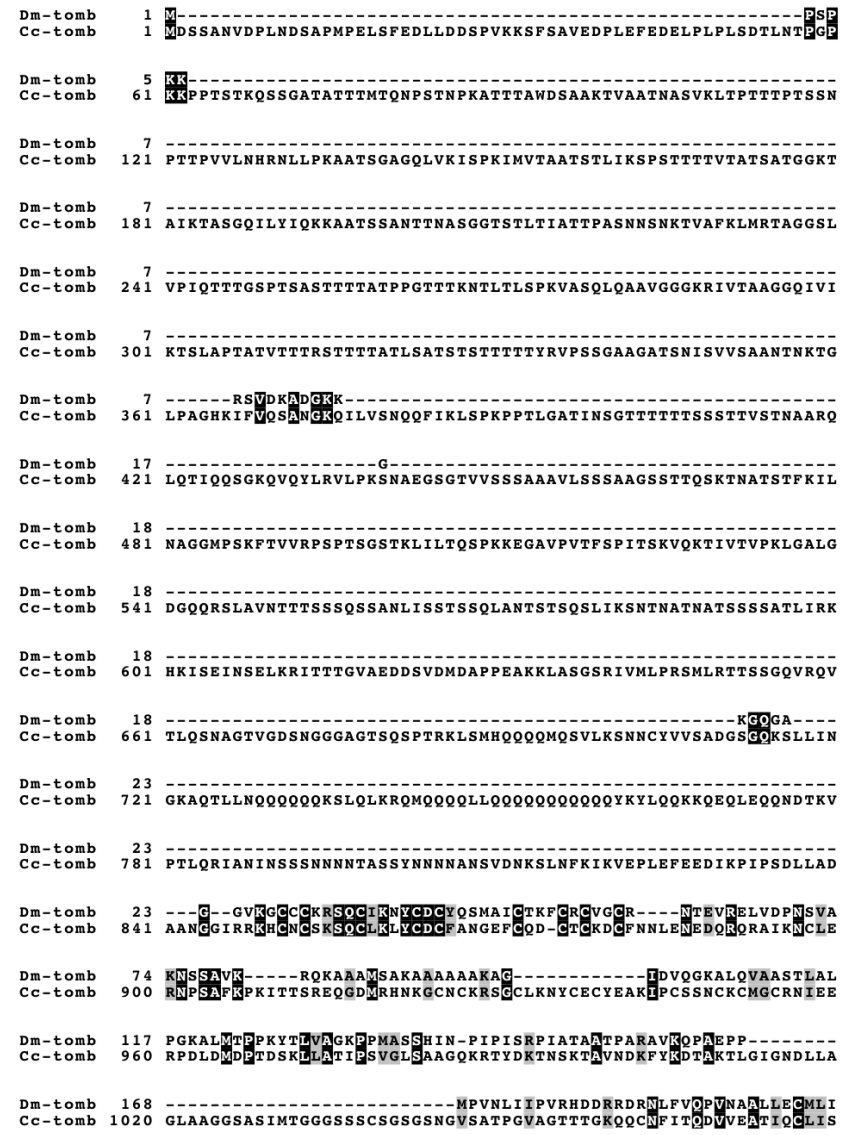

Figure 8.1 (Part 2). Alignment of *D. melanogaster* meiotic arrest genes (tMAC) and their homologues in Medfly. (**A**) *tomb*. Homology was not particularly strong and the Dm sequence was much shorter. However, both are lin-54 family proteins, and Cc-tomb possesses the CRC and CXC domains present in Dm-tomb.

A

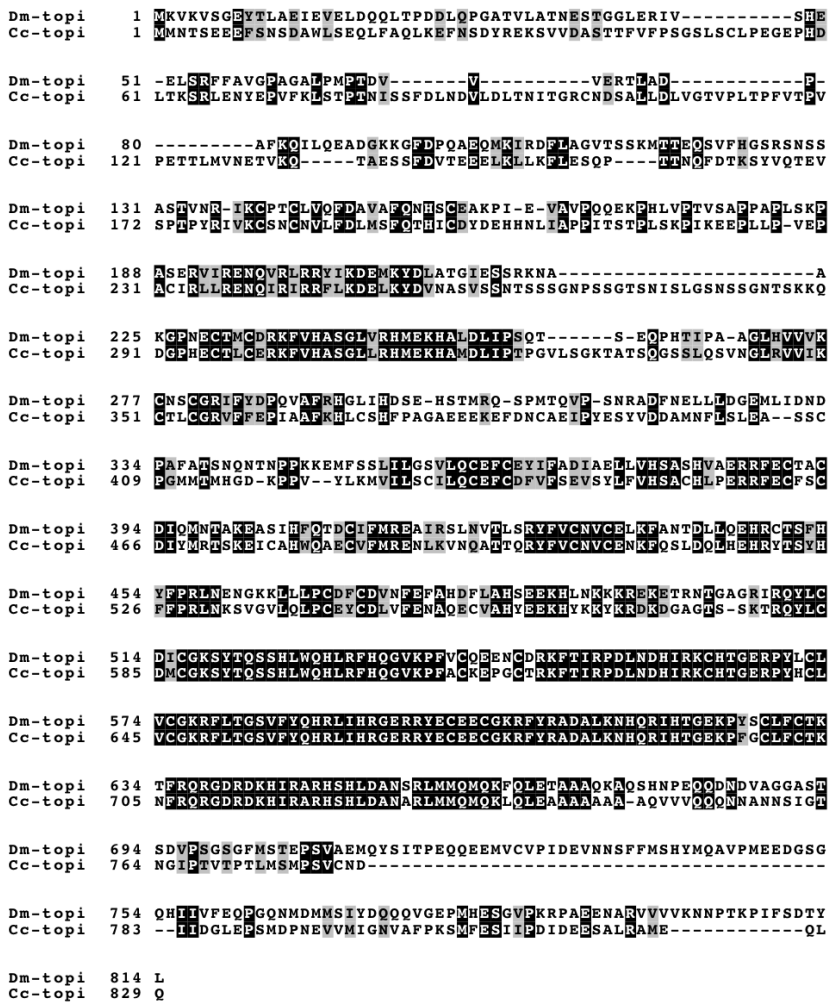

Figure 8.1 (Part 3). Alignment of *D. melanogaster* meiotic arrest genes (tMAC) and their homologues in Medfly. (**A**) *topi*.

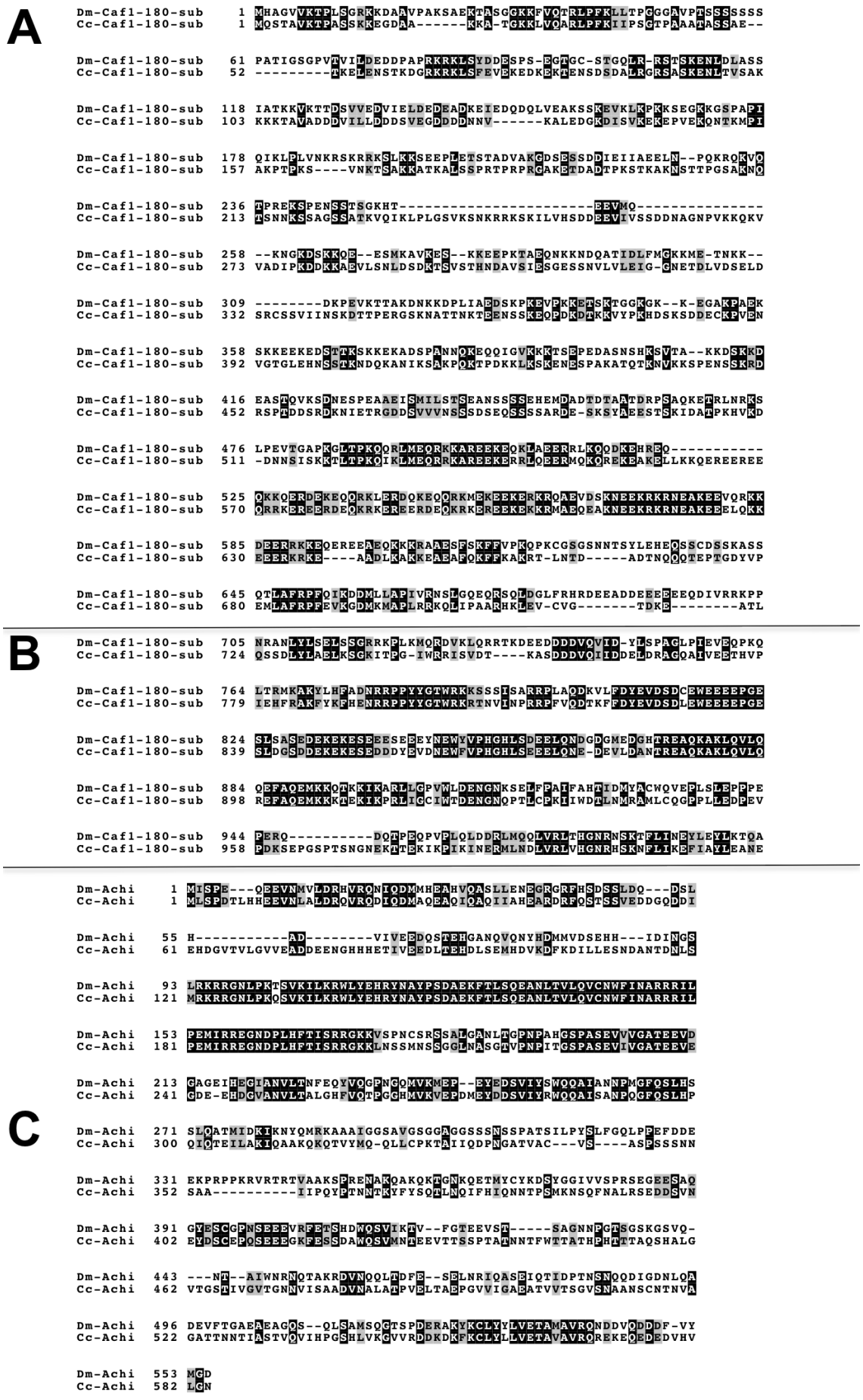

Figure 8.1 (Part 4). Alignment of *D. melanogaster* meiotic arrest genes (tMAC) and their homologues in Medfly. (**A**) *Caf1* (180 kda subunit). (**B**) *Achi*.

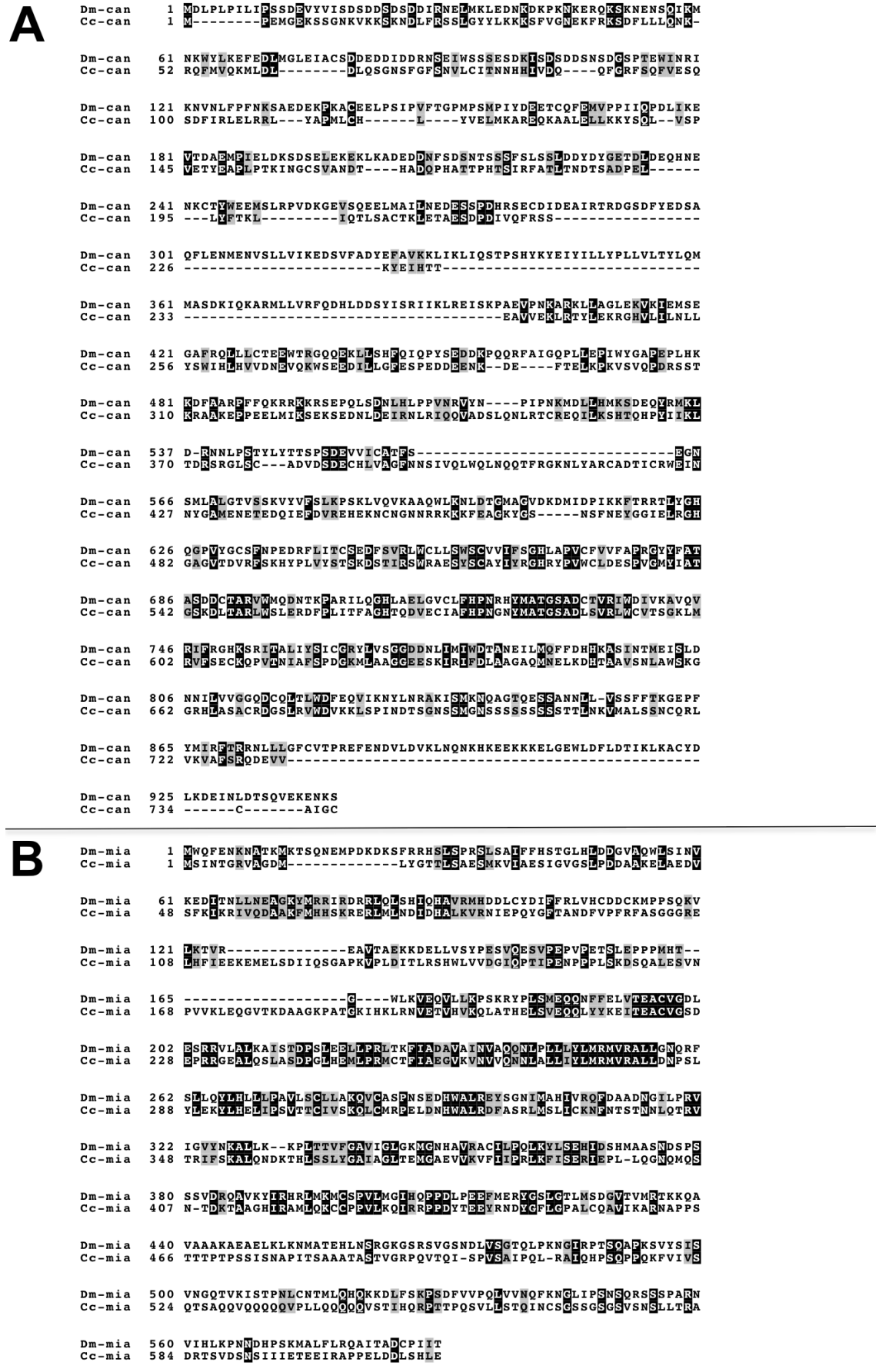

Figure 8.1 (Part 5). Alignment of *D. melanogaster* meiotic arrest genes (tTAFs) and their homologues in Medfly. (**A**) *can*. (**B**) *mia*. Cc-*Can* was annotated as a testis-specific homologue. It is unclear if Cc-*mia* is the basal or testis-specific TAF..

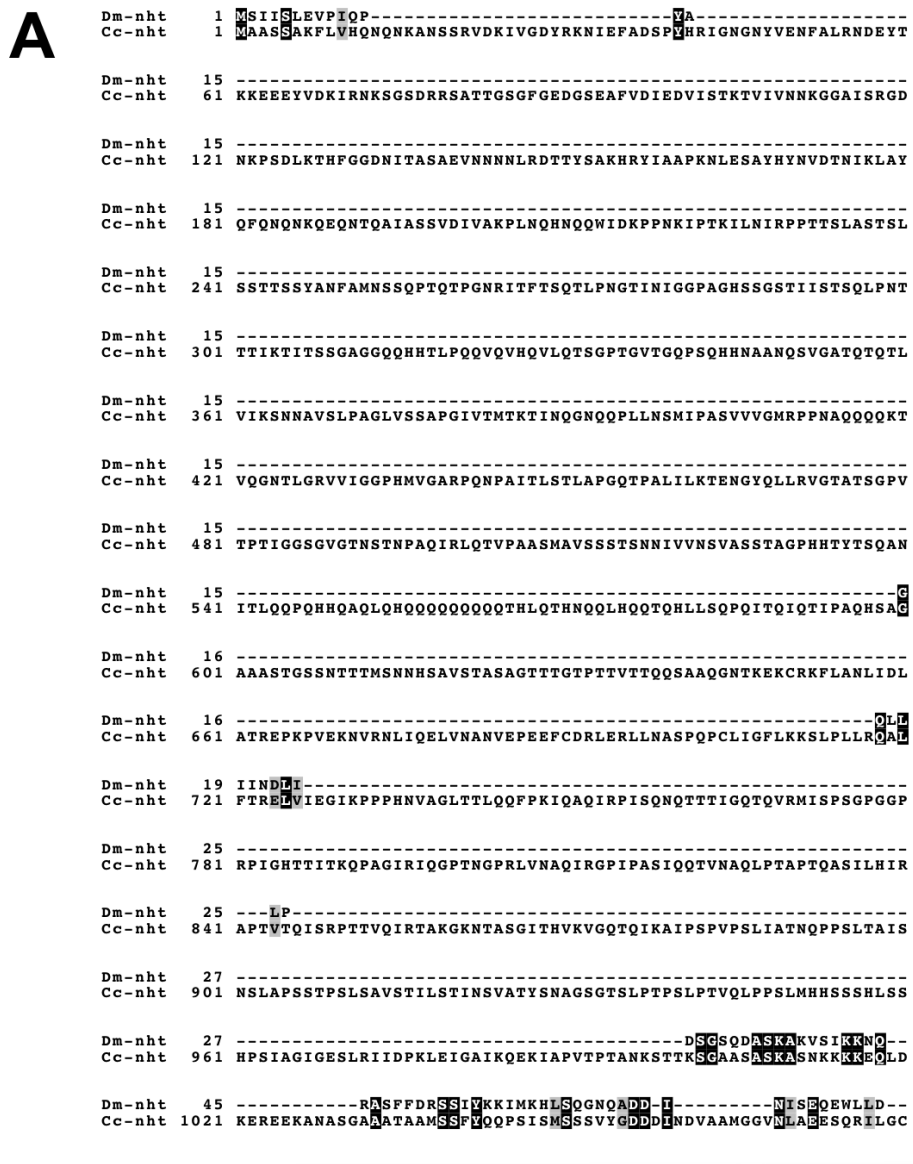

Figure 8.1 (Part 6). Alignment of *D. melanogaster* meiotic arrest genes (tTAFs) and their homologues in Medfly. (**A**) *nht*. (**B**) *sa*. It is unclear if *Cc-nht* or *Cc-mia* represent the basal or testis-specific TAFs.

Appendix 8.2 – Primer sequences used in PCR and DNA sequencing, PCR amplicons generated in the study, and CRISPR sgRNA target sites

Table 8.1 Sequences of PCR and sequencing primers

Name	Target	Sequence (5'-3')
Adapter 1	Flanking adapter	gtgtagcgtgaagacgacagaa
Adapter 2	Flanking adapter	gacgacagaaagggcgtgtgg
SS1131	Adh	gaaagctgttcgggcttcaggc
SS1132	Adh	cttggaggtgatgtcgaatttggtg
SS1693	Cc-Rp17S	tcgcaagttcgtggtatttctatc
SS1694	Cc-Rp17S	caagcaatttcaacatctcctttg
SS1713	sgRNA synthesis	aaaagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatttctagctctaaaac
SS1714	dsRed2	gaaattaatacgactcactataggccacgagttcgagatcgagttttagagctagaaatagc
SS1715	sgRNA synthesis	gaaattaatacgactcactataggtacaccttggagccgtacgttttagagctagaaatagc
SS1765	Cas9	agctggtgcagacctacaaccag
SS1853	Cas9	cggttatccttcaggaacgg
SS1928	sgRNA synthesis	gaaattaatacgactcactatagggtggcgaccggtttgcgcgttttagagctagaaatagc
SS1929	sgRNA synthesis	gaaattaatacgactcactataggcggtacttcatggtcatcgttttagagctagaaatagc
SS1930	sgRNA synthesis	gaaattaatacgactcactataggccaaggagatgaccatgagttttagagctagaaatagc
SS1931	sgRNA synthesis	gaaattaatacgactcactataggccggtgatcacgaacttggttttagagctagaaatagc
SS1932	sgRNA synthesis	gaaattaatacgactcactatagggaaagataatcatattgtgttttagagctagaaatagc
SS1933	sgRNA synthesis	gaaattaatacgactcactataggaaagataatcatattgtggttttagagctagaaatagc
SS1934	sgRNA synthesis	gaaattaatacgactcactataggaagataatcatattgtgagttttagagctagaaatagc
SS1935	sgRNA synthesis	gaaattaatacgactcactataggttgtgacgtacgttaaaggttttagagctagaaatagc
SS1936	sgRNA synthesis	gaaattaatacgactcactataggatatgattatctttctaggttttagagctagaaatagc
SS1937	sgRNA synthesis	gaaattaatacgactcactataggacgtacgtcacaatatgagttttagagctagaaatagc
SS2045	sgRNA synthesis	gaaattaatacgactcactataggtgatgacgttctcggagggttttagagctagaaatagc
SS2047	sgRNA synthesis	gaaattaatacgactcactataggagcgcgtgatgaacttcggttttagagctagaaatagc
TD95	dsRed2	cttggccatgtagatggacttgaactcc
TD96	zsGreen	caagcaggccatcaacctgtgc
TD97	zsGreen	gacttggccttgtacacggtgtcg
TD692	*piggyBac* 5'	agtcacgtaaaagataatcatgcg

TD693	*piggyBac* 3’	gtgccaaagttgtttctgactgac
TD775	dsRed2	cacaacgaggactacaccatcg
TD805	zsGreen	acgatgtcctgggggtactc
TD1018	Scraps intron	ccgttcttttctgggttcttcc
TD1085	dsRed2	acttcatccagcacaagctgacc
TD1156	pJET	atcaactgctttaacacttgtgc
TD1157	pJET	aaagaagaacatcgattttccatg
TD1167	OX4718 near pB	gctccagcttttgttccctttagc
TD1168	attP220	ggcttcggtgtgtccgtcag
TD1406	dsRed2	ccatggtcttcttctgcatcac
TD2121	dsRed2	tgtcattgtatcagtggatcggtg
TD2908	FokI	cggtagccgtacaccttcatg
TD2955	dsRed2	tgtgtgacgatgaggttgctg
TD3434	FokI	tcgtggataccaaggcctac
TD3435	FokI	ttgcagttggtgatgtggtt
TD3477	OX4718 3’ flank	gagatgatggaccctctctaacca
TD3478	OX4718 3’ flank flanking	ctcgcactgtcaactgctgatc
TD3479	OX4718 3’ flank flanking	atgactcccgcattagtattatctgc
TD3685	OX4718 5’ flank flanking	ggttcaaactttacatgtgagcaatc
TD3686	OX4718 5’ flank flanking	tctaagcgtcaactgaagatttgg
TD3687	OX4718 5’ flank flanking	gttagtctaagcgcctcgttaagttc
TD3883	*piggyBac* 5’ (inverse PCR)	gacgcatgattatcttttacgtgac
TD3884	*piggyBac* 5’ (inverse PCR)	tgacacttaccgcattgaca
TD3885	*piggyBac* 5’ (inverse PCR)	gcgatgacgagcttgttggtg
TD3886	*piggyBac* 5’ (inverse PCR)	tccaagcggcgactgagatg
TD3887	*piggyBac* 3’ (inverse PCR)	caacatgactgtttttaaagtacaaa
TD3888	*piggyBac* 3’ (inverse PCR)	gtcagaaacaactttggcacatatc
TD3889	*piggyBac* 3’ (inverse PCR)	cctcgatatacagaccgataaaac
TD3890	*piggyBac* 3’ (inverse PCR)	tgcatttgcctttcgccttat
TD3938	Ccprot1	cattccttggcaccgcgtcgta
TD3939	Ccprot1	ataccgctgggacgttgac
TD3940	Ccprot2	agctttgctccttgtcgcactg
TD3941	Ccprot2	gccgacagtccacagcatttc
TD3947	Dmprot1	aatgagtgcaagagcctgtg

TD3948	ZsGreen	gtcaggtgccacttctggtt
TD3950	Cc-RpP0	ccgcctggaaagctcaata
TD3951	Cc-RpP0	gagctggagcgcgtacttt
UFP1	5’ RACE adapter	ctaatacgactcactatagggcaagcagtggtatcaacgcagagt
UFP2	5’ RACE adapter	ctaatacgactcactatagggc

Table 8.2 Amplicons generated by standard, inverse, colony and RACE PCR

Type	Chapter	Target	Primers		Product size (bp)	Notes
Standard	3	OX4718 (left transposon)	TD693	TD1167	485	Immobilisation of the OX4718 transposon
		OX4718 (central element)	TD1168	TD692	251	
		OX4718 (right transposon)	TD2908	TD775	551	
		gDNA flanking OX4718	TD3685	TD3477	Varied	Large polymorphisms were present in the flanking sequence of certain individuals
			TD3685	TD3478		
			TD3685	TD3479		
			TD3686	TD3477		
			TD3686	TD3478		
			TD3686	TD3479		
			TD3687	TD3477		
			TD3687	TD3478		
			TD3687	TD3479		
	6	dsRed2	TD2121	TD1085	1438	Identification of CRISPR-induced mutations
		adh	SS1131	SS1132	491	
		zsGreen	TD1018	TD805	364	
		dsRed2	TD2121	TD95	935	
Inverse		piggyBac 5’	TD3883	TD3884	Varied with restriction digests	First 5’ reaction
		piggyBac 5’	TD3885	TD3886		Second 5’ reaction
		piggyBac 3’	TD3887	TD3888		First 3’ reaction
		piggyBac 3’	TD3889	TD3890		Second 3’ reaction
Colony	Multiple	pJET	TD1156	TD1157	> 174	Size varied with the insert ligated into pJET
5’RACE	4	Ccprot1	UFP mix (UFP 1 & 2)	TD3938	416	First nested PCR
				TD3939	317	Second nested PCR
		Ccprot2		TD3940	286	First nested PCR
				TD3941	256	Second nested PCR

Table 8.3 Amplicons generated by RT-PCR and qRT-PCR

Type	Chapter	Target	Primers		Product size		Notes
					cDNA	gDNA	
RT	4	zsGreen	TD3947	TD3948	1200		Dmprot1 promoter functional in OX5140
	6	zsGreen	TD96	TD97	447		3xp3-zsGreen is transcribed in OX5154 but not adequately

							translated
		Cas9	SS1765	SS1853	751		Cas9 is maternally deposited to embryos in OX5173
	4, 6	Cc-RpP0	TD3950	TD3951	315	542	Internal standard
	4	Adh	SS1131	SS1132	329	491	Internal standard
qRT	5	Ccprot1-FokI	TD3434	TD3435	232		Tetracycline partially represses tetO-Ccprot1-FokI in OX5195(2)AU1
		Cc-Rp17S	SS1693	SS1694	115		Internal standard

Table 8.4 CRISPR sgRNAs used in the study

Name	Target	sgRNA sequence (5'-3')	Activity confirmed?
SS2045	dsRed2	ggtgatgacgttctcggagg	Confirmed in vivo
SS2047		ggagcgcgtgatgaacttcg	
SS1928	zsGreen	gggtggcgaccggtttgcgc	None in vivo or in vitro
SS1929		ggcggtacttcatggtcatc	
SS1930		ggccaaggagatgaccatga	
SS1931		ggccggtgatcacgaacttg	Confirmed in vivo and in vitro
SS1932	pB 3'	gggaaagataatcatattgt	Confirmed in vitro but not in vivo
SS1933		ggaaagataatcatattgtg	
SS1934		ggaagataatcatattgtga	
SS1936		ggatatgattatctttctag	
SS1935		ggttgtgacgtacgttaaag	None in vitro
SS1937		ggacgtacgtcacaatatga	

The two 5' nucleotides of the sgRNA were always GG (to facilitate efficient transcription from the T7 promoter); this did not match the genomic target in most instances.

Appendix 8.3 – Transgenic expression constructs investigated in this study and a summary of the observed phenotypes after transgenesis

Table 8.5: Summary of all expression constructs tested in this study

Chapter	#	Vector type	Function	Phenotype observed
3	OX3671	2-pB	Fluorescent sperm marking, validating Ccβ2T or Ccβ2T[short]-Cchsp83 as a regulator of tTAV	Sperm marking weak. tTAV expressed as desired in OX4282
	OX4282			
	OX4353		Repressible male sterility	Penetrant & repressible sterility
	OX4705		Fluorescent sperm marking Repressible male sterility	Sperm marking weak Penetrant & repressible sterility
	OX4718	4-pB		
	OX4801	2-pB		Sperm marking weak Sterility not fully penetrant
	OX5036			Sperm marking weak Sterility not fully penetrant
4	OX5122		Fluorescent sperm marking.	Sperm marking strong

	OX5123			
	OX5150			
	OX5140			Sperm marking absent
	OX5182		Confirm that Dmhsp70 promoter-Ccprot1 5'UTR mediated transcription of zsGreen in with translational delay in spermatids	Strong reporter translation, appropriately delayed
	OX5184			
	OX5186			
5	OX5195	4-pB	Fluorescent sperm marking Repressible male sterility	Sperm marking strong Penetrant sterility but not repressible
	OX5241			
	OX5242			Sperm marking strong Penetrant sterility, mostly repressible
	OX5257			
6	OX4014	2-pB	Marker expressing line used to validate CRISPR and TALEN systems for targeted mutagenesis	
	OX5134		Germline expression of nanos-Cas9	No lines isolated (3xp3-zsGreen not translated)
	OX5154		Two-marker construct to confirm that 3xp3 promoter does not function in Medfly	3xp3-zsGreen was not visibly translated, despite transcription
	OX5173		Germline expression of nanos-Cas9	High efficiency targeted CRISPR mutagenesis
	OX3022	pB helper	Plasmid DNA (OX3022) or mRNA (OX3081) expression constructs for piggyBac transposase.	
	OX3081			
	OX3133	2-pB	Germline expression of piggyBac transposase (to immobilise four-ended piggyBac insertions)	

Appendix 8.4 – U6 promoter sequences suitable for germline expression of sgRNAs and CRISPR sgRNAs active against piggyBac ITRs

Materials for these experiments were prepared, but there was insufficient time to perform them. It was demonstrated that expression of sgRNAs in the germline (under control of the U6 promoter) increased the efficiency of gene editing (Kondo and Ueda, 2013). Two U6 promoter homologues from Medfly are presented, which could mediate the development of this genetic tool. It would have also been interesting to validate the CRISPR platform to mediate piggyBac end removal. sgRNAs with validated activity against the piggyBac 3' ITR were characterised. These results are summarised in **Figure 8.2**.

A

Cc-U6 promoter [1]

ACTCAAAAACAAATGATCCGATCACTATCGAATTTTTTTACATATTCTATATATAGTTCTCCCTACGATGACCTATCGAT
TTTTGAACTGATCTGAAATCGGATTTTAGCAGGCCAAAACAACCAAAATTTTGGGTAAAATTCGACTATTTTATTTAACA
CGCCGCCATATTGTAAATTTTTTATTTTTTTATCGTAAGTCATTGTACAGTCAATATTAACTAGTAGAGAATTTTTTTTT
ACAGATTTCATCCACGGAGAAGGCCGGAATCACGGCAGCAGTGAAGGACCATTTTTCTCGCGCATATAAACGCATGTATT
TCACAACAATAATAAAGCAACCATTGTTGCTTGTTTGCTTGCTCACACTTTGCCAACCCTCAGCATACGTATAATTCGGA
ACTCACTACGATTTCGTTTATCACATTGTGCTGTCTGCAATTCACAGATGCTTTTTCGGCAATTGGCTGCTATAAATACT
TTGTGTTTTTAATGTGATTT**G**

Cc-U6 promoter [2]

CTTTTTATTTGTTTACTTTTGCACTCTTCATCTTCCACTGCTTAACACTCAAGGTGTAATATCAAACATCAAAATTACTT
GCTTGCCACTATTCTACGCAAAAGAAAAAATATAAAAAATTTCTTGAGAGCTGAAAGCTCTGCTTAAATTTGAGAACAGA
GCACAAAATACCAAGGTTACAGCACAATGACAGTCCTTAACTTTGCGAGCAAAGAAATTTTTAAATACTTAAATGCAGCC
CTGTAAATAATGTCAGCTGTTTTTCACCAGCCACATGCTCTACTCTTCTCATATTTGCACATAAACTGCTCTCACTGCTC
TTTGCTAGCACCGAATTATACACAACGTTGGTGGTTGTGTACAGATATGTACCTGTATGTGTTCGTGTGTATGTGCATGC
GTGTATTAAAGAAATTCATTCCTGCTGAATTTTCTTGTAATTCGCATGTGCTTTTTCGTTACGTGCTCCATACAAATATG
GAAGTGAGAATGAAGTATTT**G**

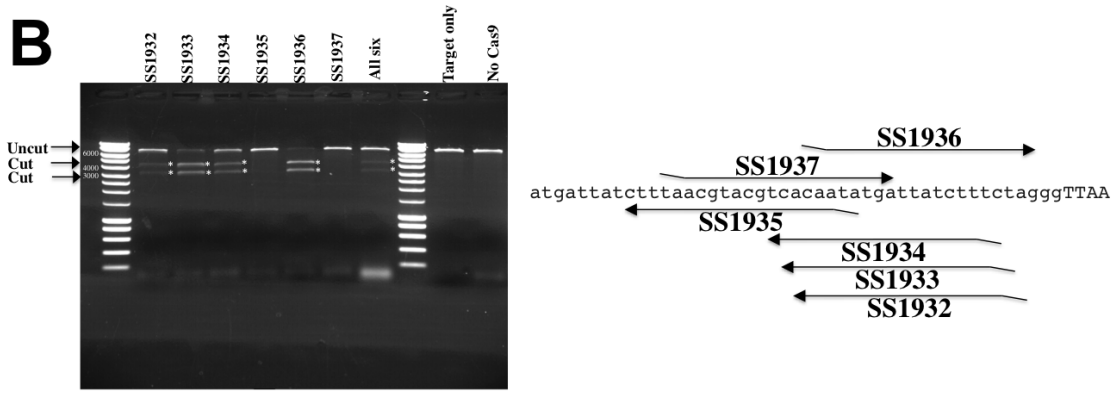

Figure 8.2. Additional materials potentially useful for CRISPR modification of Medfly. (**A**) Two putative Medfly U6 promoter fragment sequences (-500 to +1), which could be used to express sgRNAs in the germline, and hence improve the efficiency of genetic editing. The +1 guanine of the putative transcriptional start site is shown in bold; the sgRNA sequence would be cloned at this position. (**B**) *In vitro* validation of six sgRNAs targeting the pB 3' ITR, on target plasmid DNA. Cut sizes: ~ 4200 & 3200 bp (all sites within 35 bp). SS1932-1934 & 1936 were functional. Controls were: target DNA only; and no Cas9. (**C**) Target sites within piggyBac 3'. In all instances the two 5' nucleotides (GG) mismatched the target sequence.

Summary

Medfly (*Ceratitis capitata*) is an invasive Tephritid fruit fly that severely disrupts global agricultural productivity. Pesticides are the primary control method despite genetic resistance, questionable efficacy, and negative effects upon the environment. The sterile insect technique (SIT) is an ecofriendly alternative, that suppresses the reproduction of wild Medfly by the mass release of sterilised males. Currently, males are sterilised by irradiation, which frequently reduces the ability of males to court females and thereby suppress reproduction.

To address these undesirable effects, we developed a novel sterilisation strategy, based on the tetracycline-repressible expression of a nuclease effector in the male germline. Strains expressing these effectors were 99-100% sterile in the absence of tetracycline, but fertile in the presence of tetracycline. Male mating competitiveness was not detectably reduced in one strain expressing the effector, indicating that these expression systems are suitable for field use. Subsequently, a fluorescent marking system to label sperm was developed, which provided a useful tool to assess the mating competitiveness of sterile males: it was possible to accurately differentiate whether females had mated wild or transgenic males, under field-simulated conditions.

These components may be merged with a tetracycline-repressible genetic switch to remove females from the rearing population, to improve the efficacy of the strategy by releasing sterile males alone. Thereafter, a full assessment of the life history traits of the strain and its mating competitiveness under field-like conditions will be performed, to confirm that the release of these sterile males is capable of suppressing wild populations of Medfly.

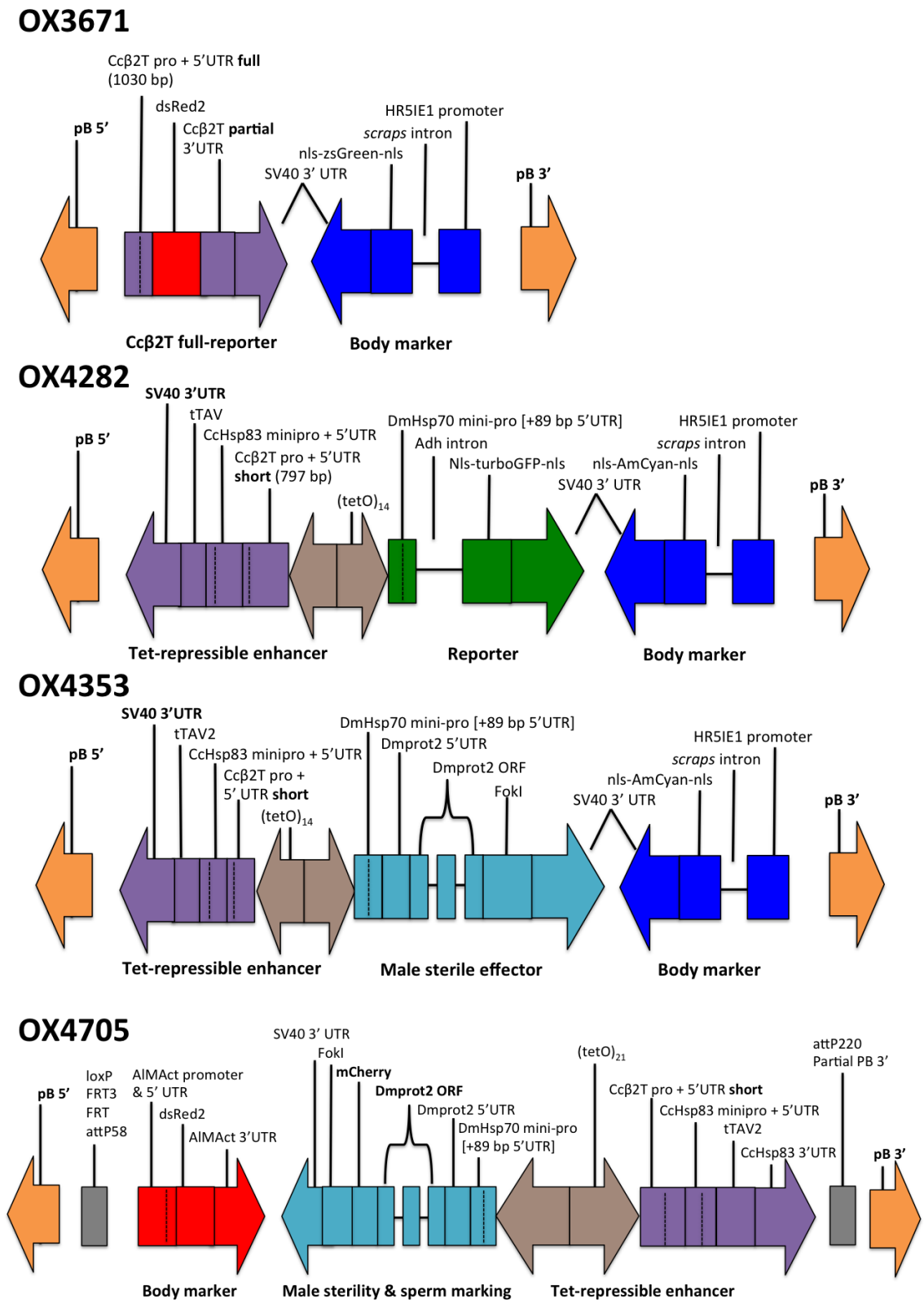

Figure 2.2 (Part 1). Expression constructs used in the study. All diagrams are repeated in the relevant results section with a full description of their contents. OX3671 & OX4282 were reporter constructs to test Ccβ2tubulin-derived expression systems. Pre-meiotic transcription and translation of tTAV in the male germline was required to activate sufficient pre-meiotic transcription of a protamine-FokI male sterility effector. The Ccβ2tubulin-Cchsp83-tTAV element from OX4282, was used in all subsequent constructs. OX4353 & OX4705 were constructs for tetracycline-repressible male sterility. The OX4705 male sterility effector has a fused fluorescent sperm marking system (without SG4 linkers).

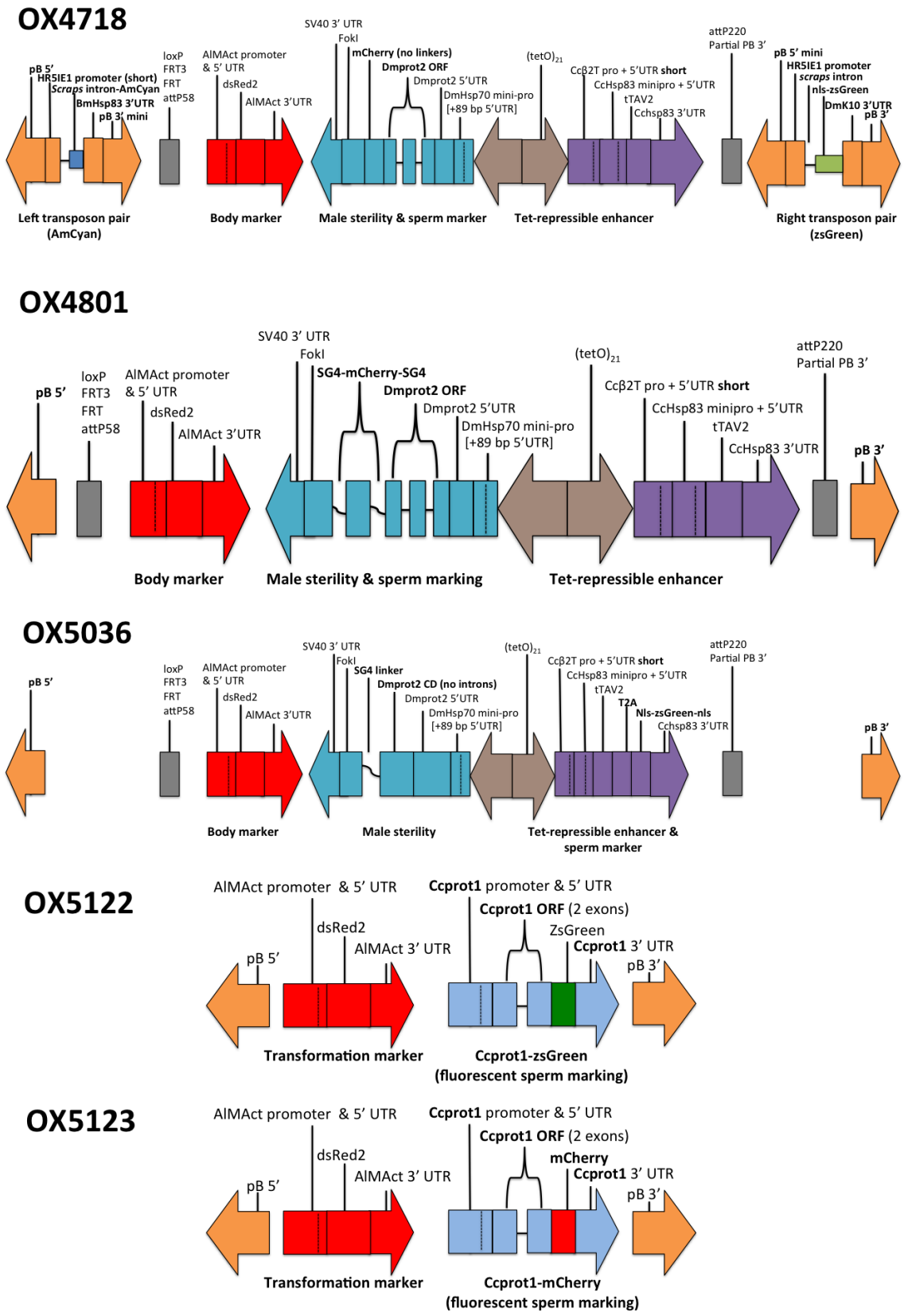

Figure 2.2 (Part 2). Expression constructs used in the study. OX4718, OX4801 & OX5036 were constructs for tetracycline-repressible male sterility with fluorescent sperm marking systems. The fluorescent sperm marker was fused to the sterility effector in OX4801 (with SG4 linkers) and OX4718 (without SG4 linkers). In OX5036, tTAV and nls-zsGreen-nls (the fluorescent sperm marking system) were translated from the same mRNA as two polypeptides, separated at the T2A sequence. OX5122 & OX5123 were constructs with protamine-fluorescent marker fusions (Ccprot1-zsGreen or Ccprot1-mCherry) for fluorescent sperm marking.

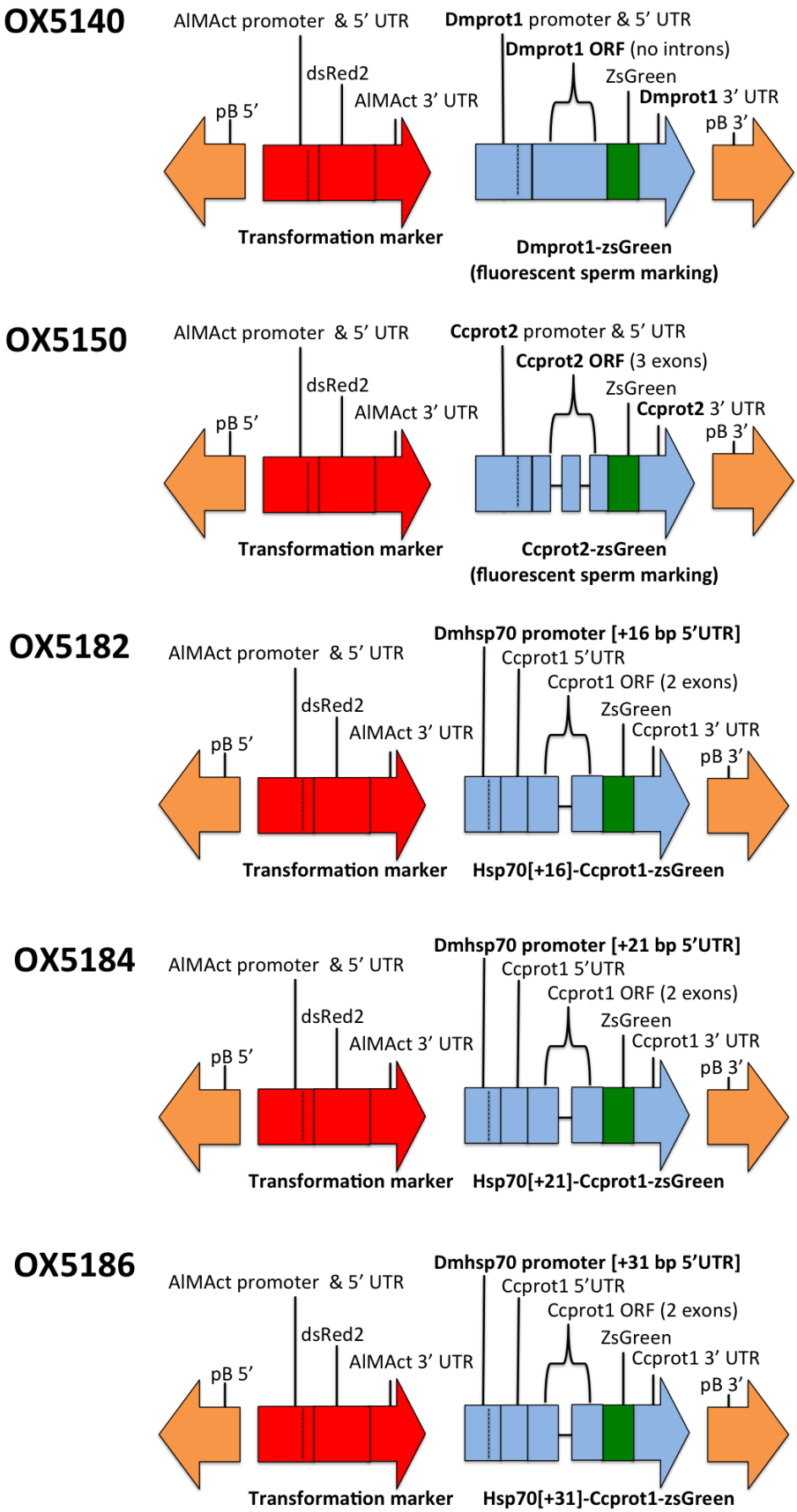

Figure 2.2 (Part 3). Expression constructs used in the study. OX5140 & OX5150 were constructs with protamine-fluorescent marker fusions for fluorescent sperm marking (Dmprot1-zsGreen or Ccprot2-zsGreen). OX5182, OX5184 & OX5186 were DmHsp70 promoter-Ccprot1-zsGreen reporter fusions to verify that the Ccprot1 5'UTR would mediate translational repression when applied to regulate effectors for repressible male sterility. They varied in the length of Dmhsp70 5'UTR included in the fusion to the Ccprot1 5'UTR.

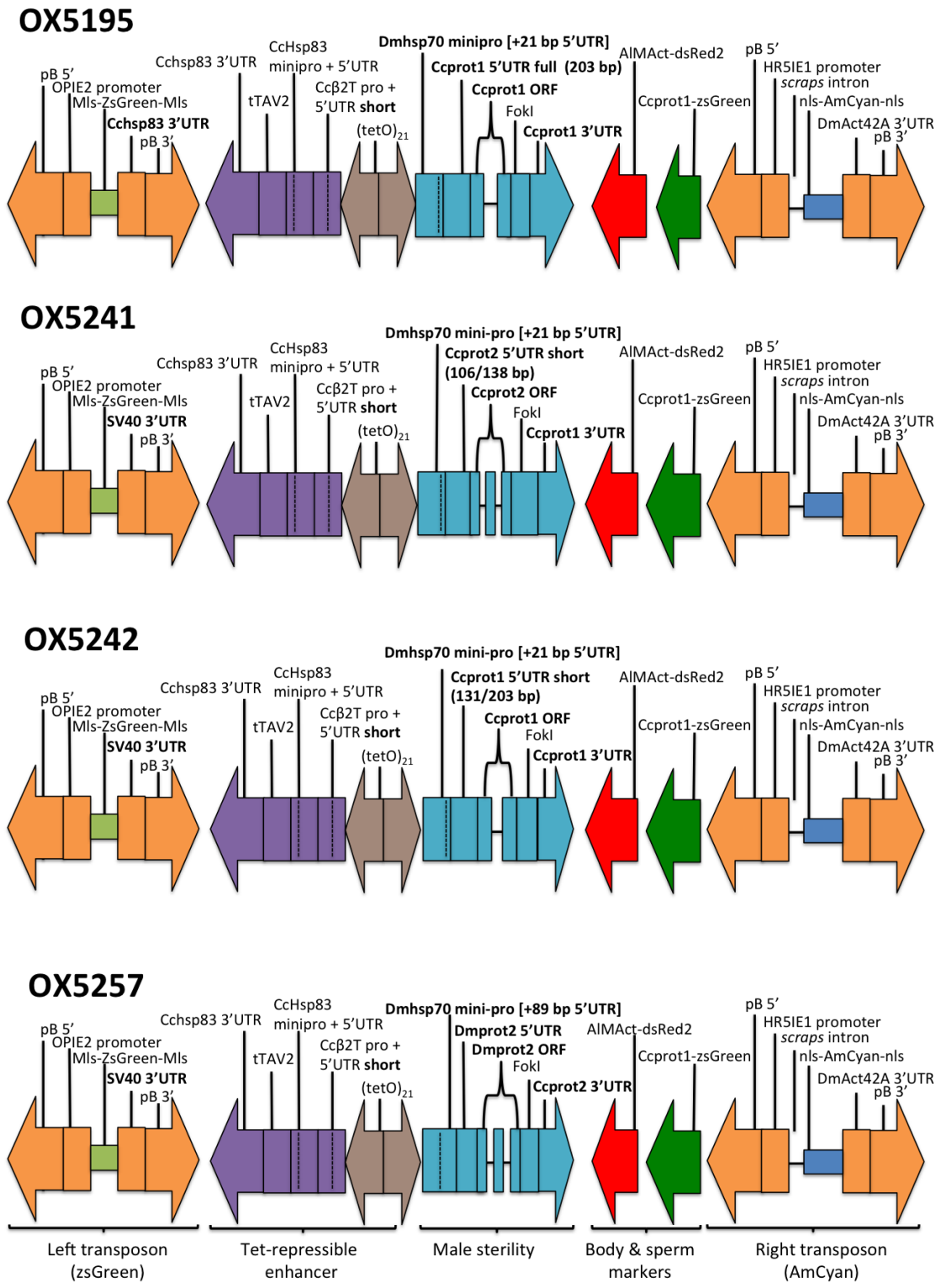

Figure 2.2 (Part 4). Expression constructs used in the study. OX5195, OX5241, OX5242 & OX5257 were constructs for tetracycline-repressible male sterility with fluorescent sperm marking systems. Four systems for repressible male sterility were tested: OX5195 (Ccprot1-full 5' UTR-FokI); OX5241 (Ccprot2-short 5'UTR-FokI); OX5242 (Ccprot1-short 5'UTR-FokI); OX5257 (Dmprot2-chimeric-new-FokI). All constructs incorporated a fluorescent marker translated in the body (AlMAct promoter-AlMAct 5' UTR-dsRed2-AlMAct 3' UTR) and a second fluorescent marker translated in sperm nuclei (Ccprot1 promoter-Ccprot1 5'UTR-Ccprot1 ORF-zsGreen-Ccprot1 3'UTR).

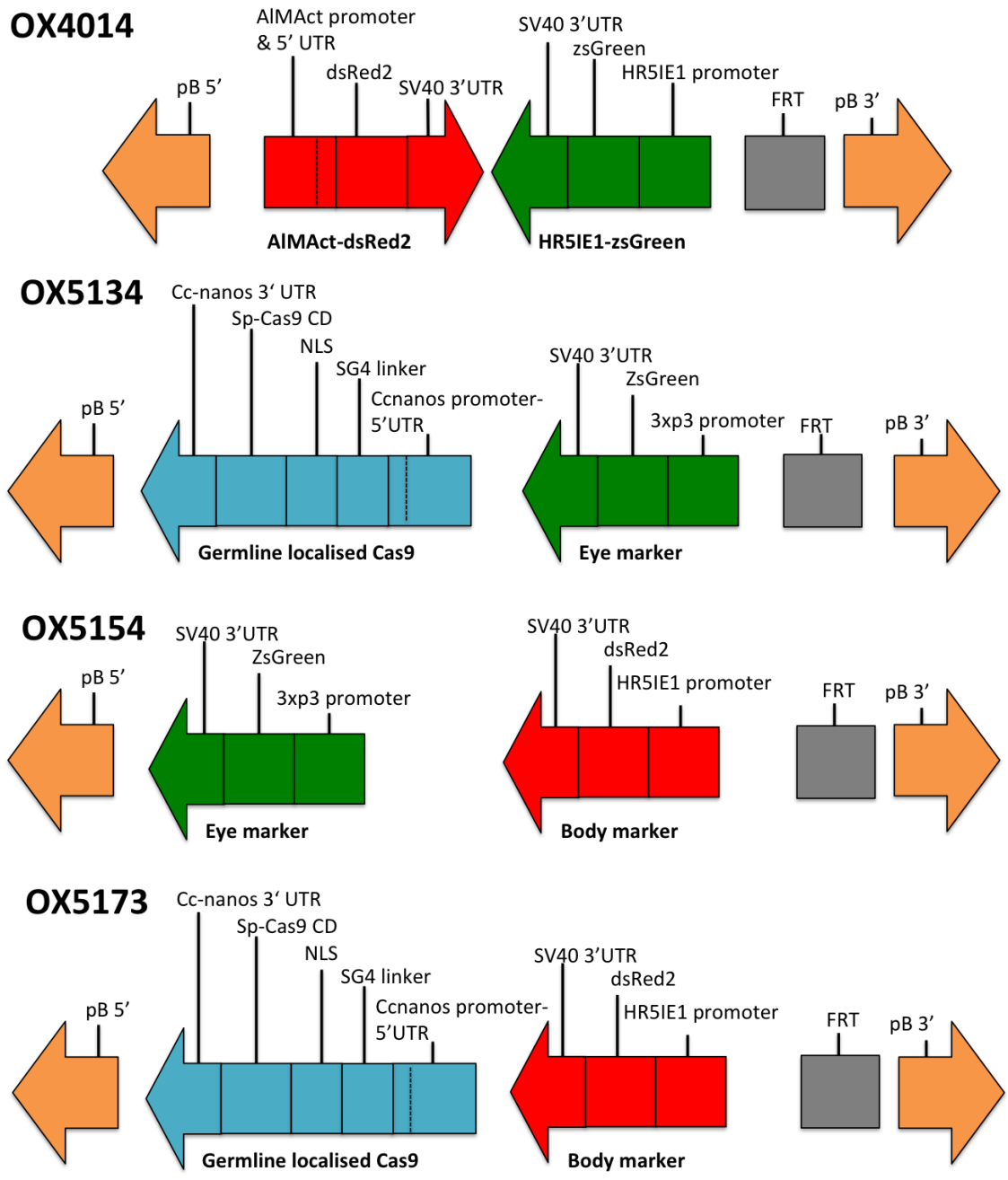

Figure 2.2 (Part 5). Expression constructs used in the study. OX4014 is a piggyBac vector with multiple fluorescent markers, used in CRISPR mutagenesis experiments. OX5134 and OX5173 were designed to express Cas9 in the germline (nanos-Cas9). Lines of OX5134 were not obtained. OX5154 was a double transformation marker construct to confirm that 3xP3-zsGreen (also used in OX5134) was not functional, therefore explaining why OX5134 transgenic lines were not obtained.

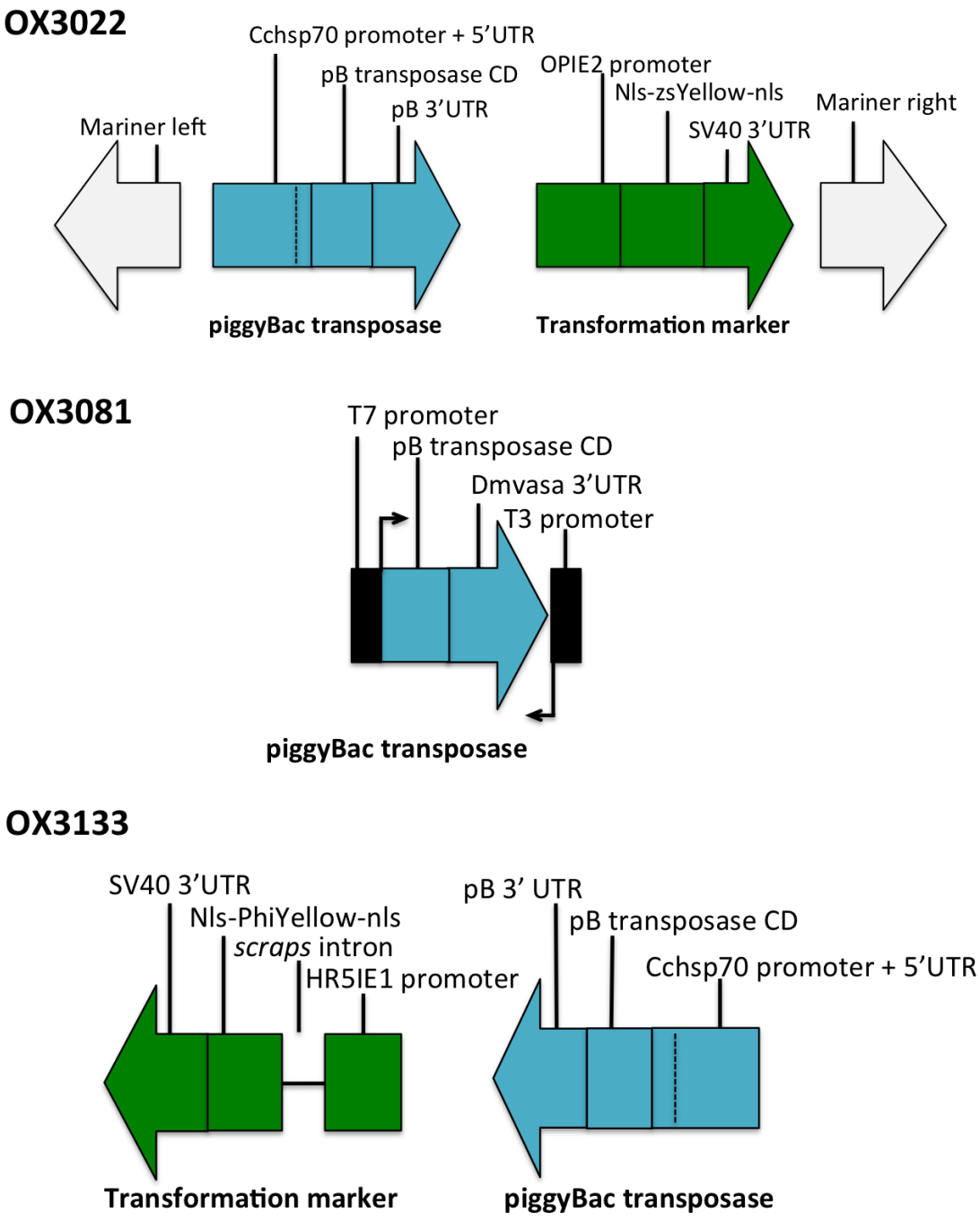

Figure 2.2 (Part 6). Expression constructs used in the study. OX3022 and OX3081 were helper constructs for piggyBac transgenesis, encoding pB transposase. OX3022 was directly injected; OX3081 was converted to mRNA by in vitro transcription from the T7 promoter. OX3022 contains transposable ends (mariner) and a transformation marker, but transgenic lines were not made for this construct. Transgenic lines of OX3133 were used to mobilise ("resolve") the piggyBac end pairs in four-ended constructs, thereby locking the central element encoding the transgenic effectors in the genome. These OX3133 lines also lack pB ends (it was a 4-ended vector, resolved by the transposase encoded on the construct).

Form: PGR_Submission_200701

NOTICE OF SUBMISSION OF THESIS FORM:
POSTGRADUATE RESEARCH

NOTICE OF SUBMISSION OF THESIS: POSTGRADUATE RESEARCH DEGREES
Please TYPE or write in BLACK ink and use BLOCK capitals

SECTION A: TO BE COMPLETED BY THE CANDIDATE AND SUBMITTED WITH THE THESIS

CANDIDATE'S LAST NAME	
CANDIDATE'S FIRST NAME(S)	
CANDIDATE'S ID NUMBER	
SCHOOL	
TITLE OF DEGREE	Please circle appropriate degree title EdD, EngD, DSW, DClinPsy, DHS, MCh, MD, MPhil, MScD by Research, PhD
FULL TITLE OF THESIS	
IS THIS A RESUBMISSION?	YES / NO
THESIS SUBMITTED FOR EXAMINATION IN	Permanent Binding ☐ Temporary binding ☐
FULL ADDRESS FOR RECEIPT OF RESULT LETTER, DEGREE CERTIFICATE AND DETAILS OF THE GRADUATION CEREMONY	**You must notify Cardiff University immediately if this address changes via:** studentrecords@cf.ac.uk
DO YOU WISH TO ATTEND THE DEGREE CEREMONY	**YES / NO**
CONTACT TELEPHONE (WITH DIALLING CODE)	
EMAIL ADDRESS	
CANDIDATE SIGNATURE	DATE

GUIDELINES ON THE COMPLETION OF:

APPLICATION FOR A BAR ON ACCESS TO A THESIS/DISSERTATION FORM

Purpose
This document provides guidance and definitions for Heads of Schools, on the correct completion of an 'Application for a Bar on Access to a Thesis/Dissertation' form, which must be used to apply for a Bar on Access to a Thesis/Dissertation for ALL postgraduate research theses and postgraduate Master's stage student dissertations.

Process
A request may be submitted to the Academic Standards & Quality Committee to place a bar on photocopying and/or access to a candidate's work for a specified period normally of no longer duration than 2 years but can be up to 5 years in exceptional circumstances.

Any recommendation for a bar on access must be made to the Academic Standards & Quality Committee by a candidate's supervisor, supported by the Head of School or his/her nominee. It shall be the responsibility of the supervisor to make the application as soon as is reasonably practicable, and normally at the time of initial registration.

The recommendation must include a statement of the grounds on which the request is being made. When a bar on access has been granted, Student Records will notify the School via email.

The regulations governing applications for a Bar on Access to a Thesis/Dissertation can be found in the Senate Regulations for Taught Postgraduate Master's Degrees – Dissertation Submission: Modular and Non-modular Programmes at
http://www.cardiff.ac.uk/regis/sfs/regs/1112acadregs/academic-regulations-handbook-201011.html

The regulations governing applications for a Bar on Access to a Thesis/Dissertation can be found in the Senate Regulations for the Presentation and Submission of Research Degree Theses.

Please note: all applications for Bars on Access are subject to the approval of the Academic Standards & Quality Committee and application forms can be downloaded from http://www.cardiff.ac.uk/regis/sfs/postgrad/

Action to be taken by School:

1. The completed form must be accompanied by a letter from the supervisor outlining the grounds for the application and, where appropriate, providing supporting documentary evidence.

2. The form must be endorsed and signed by the Head of School or his/her nominee. In signing the form the Head of School is confirming support for the recommendation for a Bar on Access.

3. The completed form and any relevant supporting documentation must be returned to:
Student Records Team, Registry, 30-36 Newport Road, Cardiff, CF24 0DE

Bar on Access May 2012

APPLICATION FOR A BAR ON ACCESS TO A THESIS / DISSERTATION

Please note that the Title and Summary of the thesis shall normally be freely available but the thesis will not be made available until after the expiry of an approved bar on access.

The bar on access shall be regarded as operative as soon as the work is submitted but the period approved is calculated from the date on which the candidate is formally notified by the University that s/he has qualified for a degree.

SECTION A: CANDIDATE'S DETAILS

SCHOOL	
CANDIDATE'S NAME	
CANDIDATE'S STUDENT NUMBER	
PROGRAMME OF STUDY	
THESIS/DISSERTATION TITLE	

SECTION B: DETAILS OF BAR ON ACCESS REQUEST
REASON

Please indicate reason, for example, there are grounds for commercial or public sensitivity, individual confidentiality or security, or to allow the author to prepare the work for publication:

PLEASE PROVIDE SUPPORTING DOCUMENTATION WHERE APPROPRIATE

TIME LIMIT

1 to 2 years normally but up to 5 years in exceptional circumstances although a Bar on Access in order to allow the author to prepare the work for publication is normally only for one year

Please indicate number of years in box below:

Bar on Access May2012

Bars on access over 2 years

Only exceptional cases can be approved for up to 5 years so please give reason if Bar on Access request is for more than 2 years

SECTION C: REQUIRED SIGNATURES

HEAD OF SCHOOL		DATE
Please Print Name		
SUPERVISOR		DATE
Please Print Name		
CANDIDATE		DATE
Please Print Name		
Name and **email address** of member of staff to be notified of the outcome of the bar on access application.		

THIS FORM CANNOT BE PROCESSED UNLESS SIGNED BY THE HEAD OF SCHOOL